Centrum pro výzkum toxických látek v prostředí

BIOMARKERS AND TOXICITY MECHANISMS

09 – Mechanisms
Nuclear Receptors

Luděk Bláha, PřF MU, RECETOX
www.recetox.cz

Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE esf

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

OP Vzdělávání pro konkurenceschopnost

UNIVERSITAS MASARYKIANA BRUNENSIS

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

NUCLEAR (Intracellular) RECEPTORS in summary

- Important physiological functions, and
- Important roles in pathologies and chemical toxicity
  - Endocrine disruption
  - Dioxin-like toxicity,etc.
- All NRs share similar structure and mechanisms of action
  - Act as direct transcription factors on DNA
- Natural ligands are small lipophilic hormones (steroids, thyroids, retinoids)
  - Role in toxicity – NR are modulated (activated/inhibited) by structurally close xenobiotics

Structure of nuclear receptor proteins

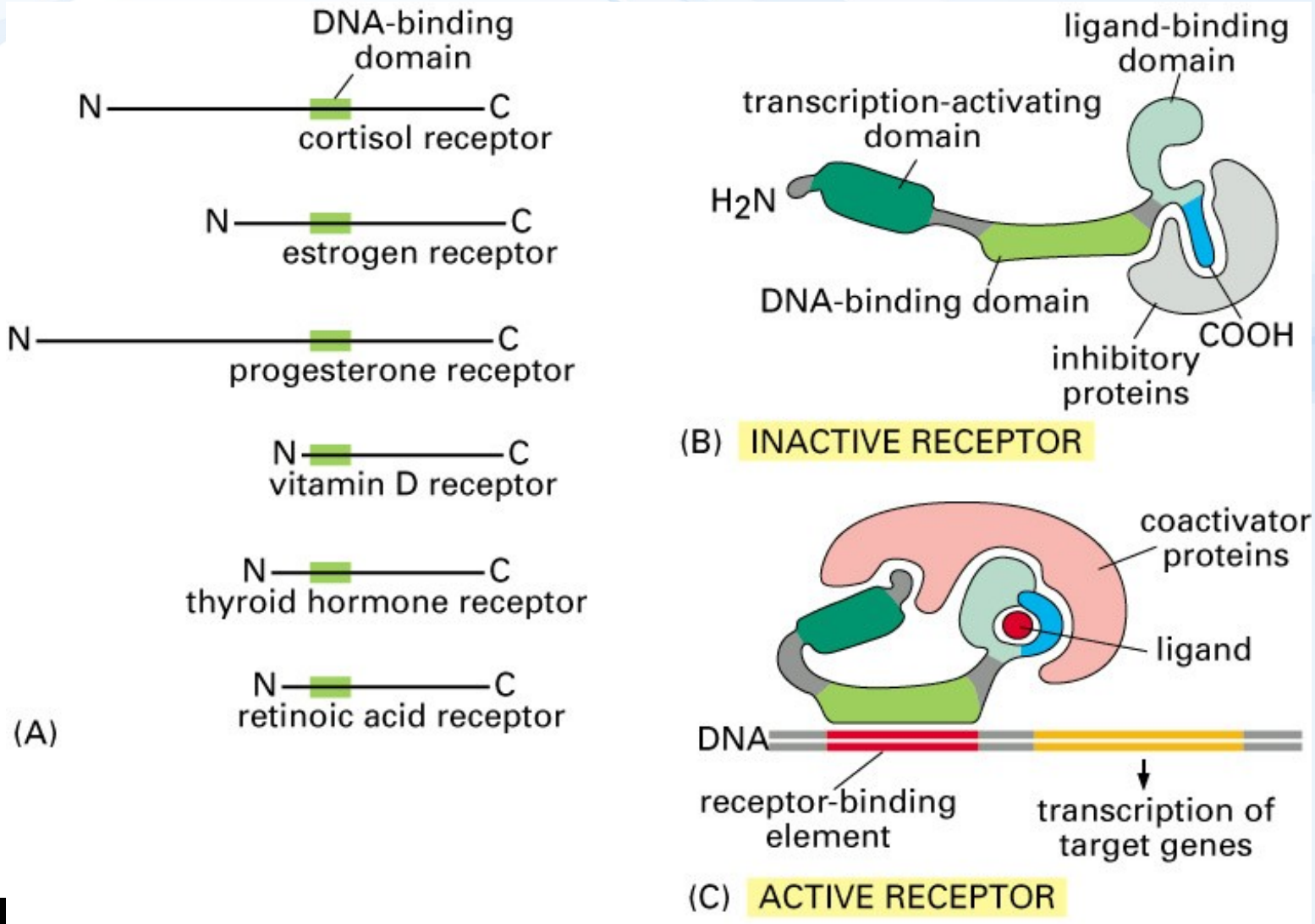
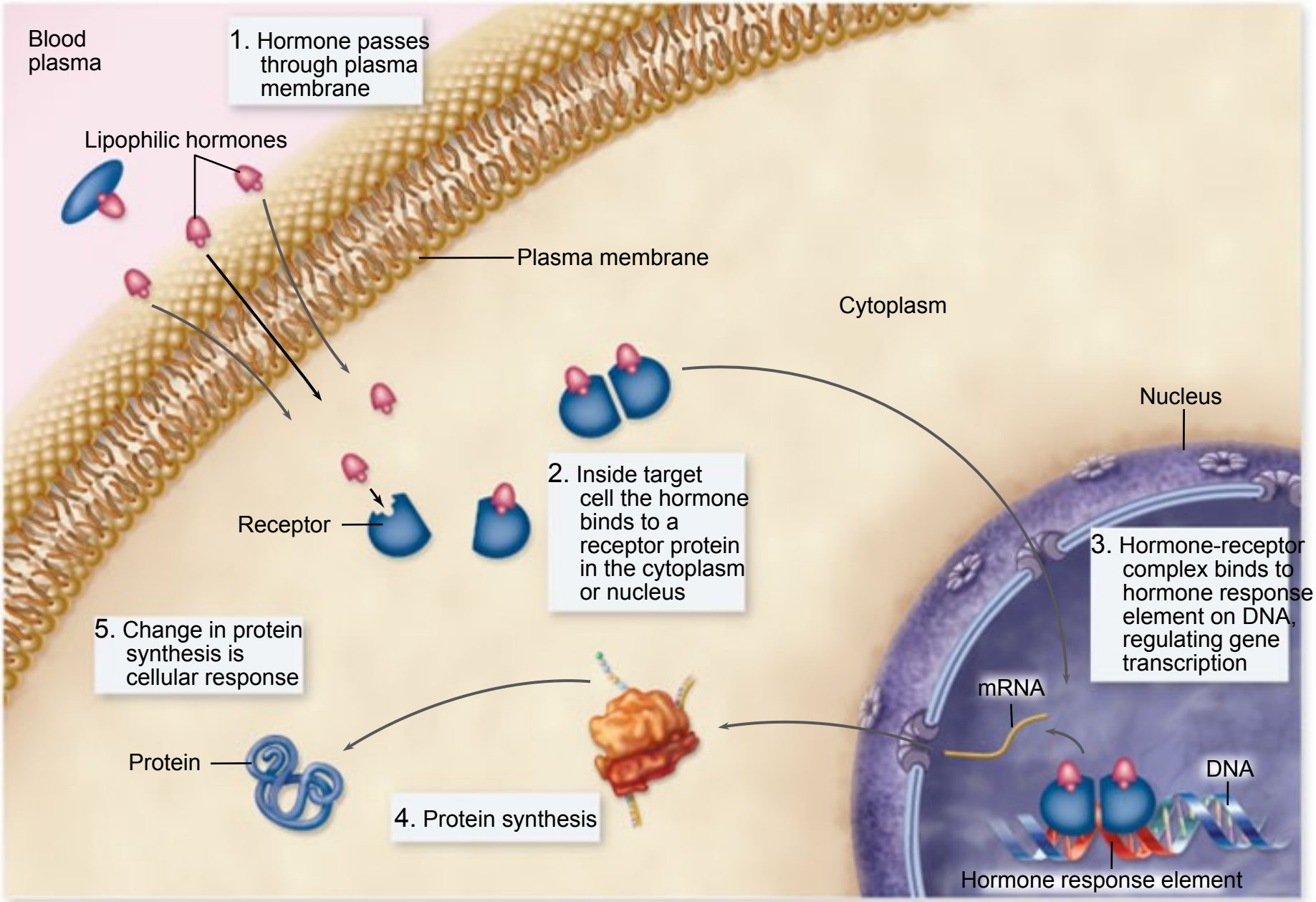


Figure 15–13 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

Fate and action of hormones activating NRs

- Circulation in the blood bound to transport proteins
- Dissociation from carrier at target cells
- Passing through cell membrane
- Binding to an intracellular receptor (either in the cytoplasm or the nucleus)
- Hormone-receptor complex binds to hormone responsive elements in DNA

→ Regulation of gene expression



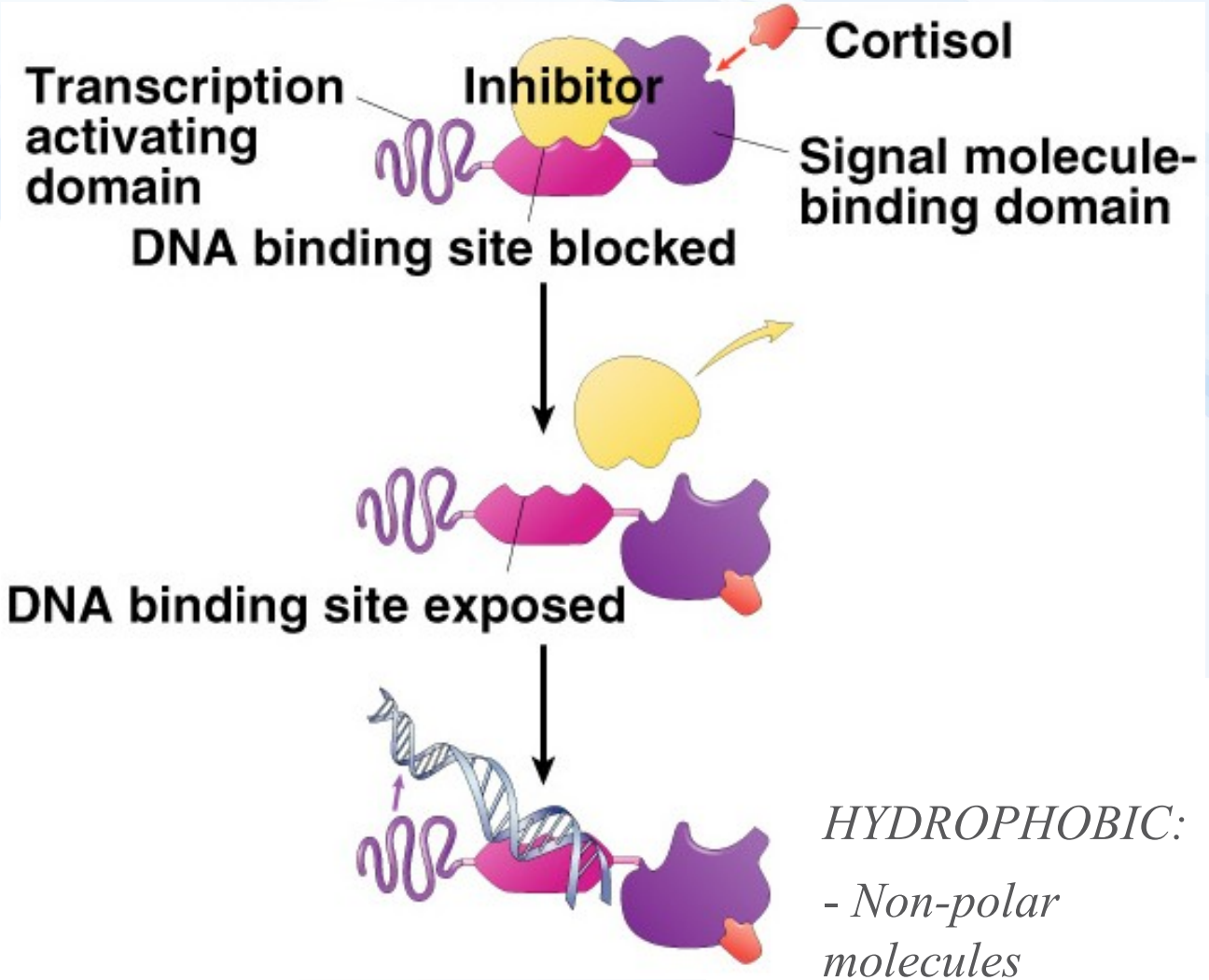
Further specificities of NRs

- **Receptor without ligand**
  - Associated with inhibitory proteins (eg Hsp90)

- Regulation of transcription activity - mechanisms may vary
  - Steroid receptors often **dimerize** with a partner to activate gene transcription
  - Receptors for vitamin D, retinoic acid and thyroid hormone form **heterodimers** and then bind to responsive elements on DNA
    - Second component of the heterodimer is RXR monomer (i.e, RXR-RAR; RXR-VDR)

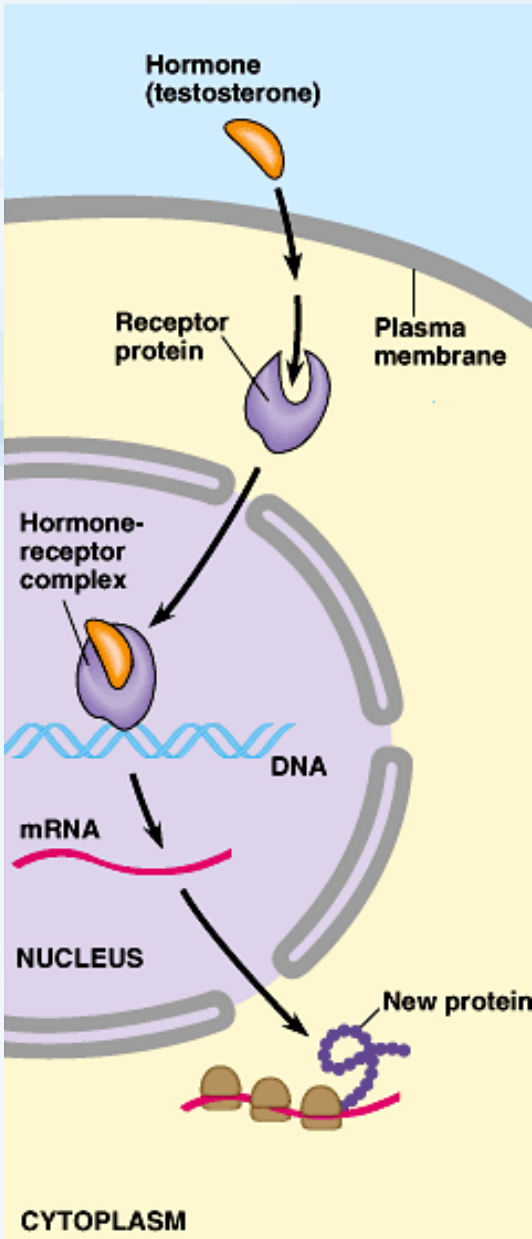
- NR dimers
  - Heterodimeric receptors - exclusively nuclear; without ligand, repress transcription by binding to their cognate sites in DNA
  - Homodimeric receptors - mostly cytoplasmic (without ligands) & hormone binding leads to nuclear translocation of receptors

General mechanism of NR action



HYDROPHOBIC:

- *Non-polar molecules*
- *Gases*
- *Steroids*



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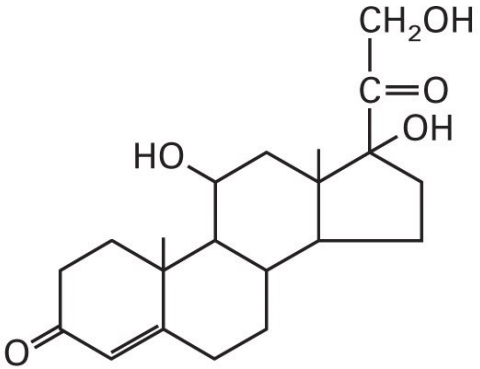
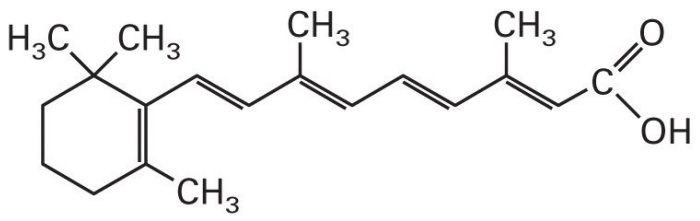
Natural ligands of NR

- **Small, lipid-soluble molecules**
  - Diffuse through plasma and nuclear membranes and interact directly with the transcription factors they control.
- STEROID HORMONES:
  - sex steroids (estrogen, progesterone, testosterone)
  - corticosteroids (glucocorticoids and mineralcorticoids)
- OTHER HORMONES and ligands

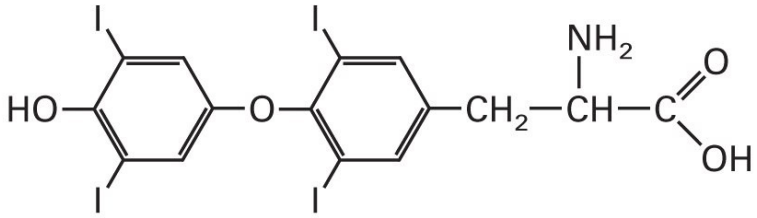
  Thyroid hormone, vitamin D3, retinoic acid, ligands of AhR



Cortisol



Retinoic acid



Thyroxine

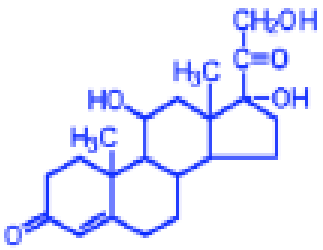
STEROIDs in more detail

Steroid hormones - a review

Steroid hormones are derived from cholesterol metabolism in mitochondria

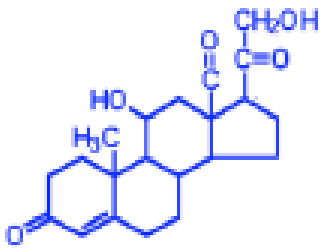
Cortisol

The dominant glucocorticoid in humans. Synthesized from progesterone in the *zona fasciculata* of the adrenal cortex. Involved in stress adaptation, elevates blood pressure and Na^+ uptake. Immunomodulation.



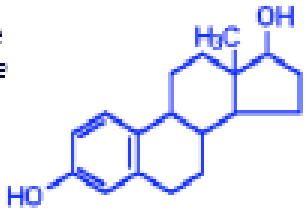
Aldosterone

Principal mineralocorticoid. Produced from progesterone in the *zona glomerulosa* of adrenal cortex, raises blood pressure and fluid volume, increases Na^+ uptake.



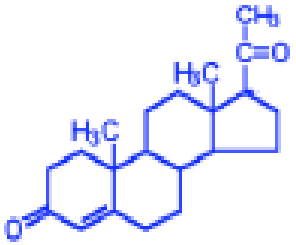
Estradiol

An estrogen, principal female sex hormone, produced in the ovary, responsible for secondary female sex characteristics. After menopause estrogen is produced from testosterone in the adrenal glands.



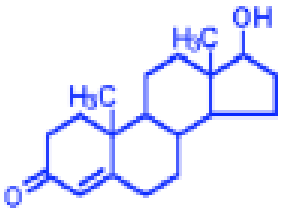
Progesterone

Produced from pregnenolone and secreted from the corpus luteum. Responsible for changes associated with luteral phase of the menstrual cycle, differentiation factor for mammary glands



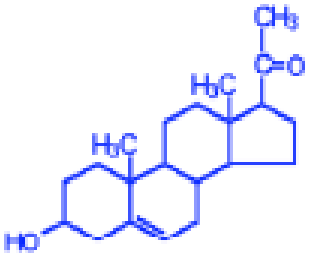
Testosterone

An androgen, male sex hormone synthesized in the testes from progesterone. Responsible for secondary male sex characteristics.

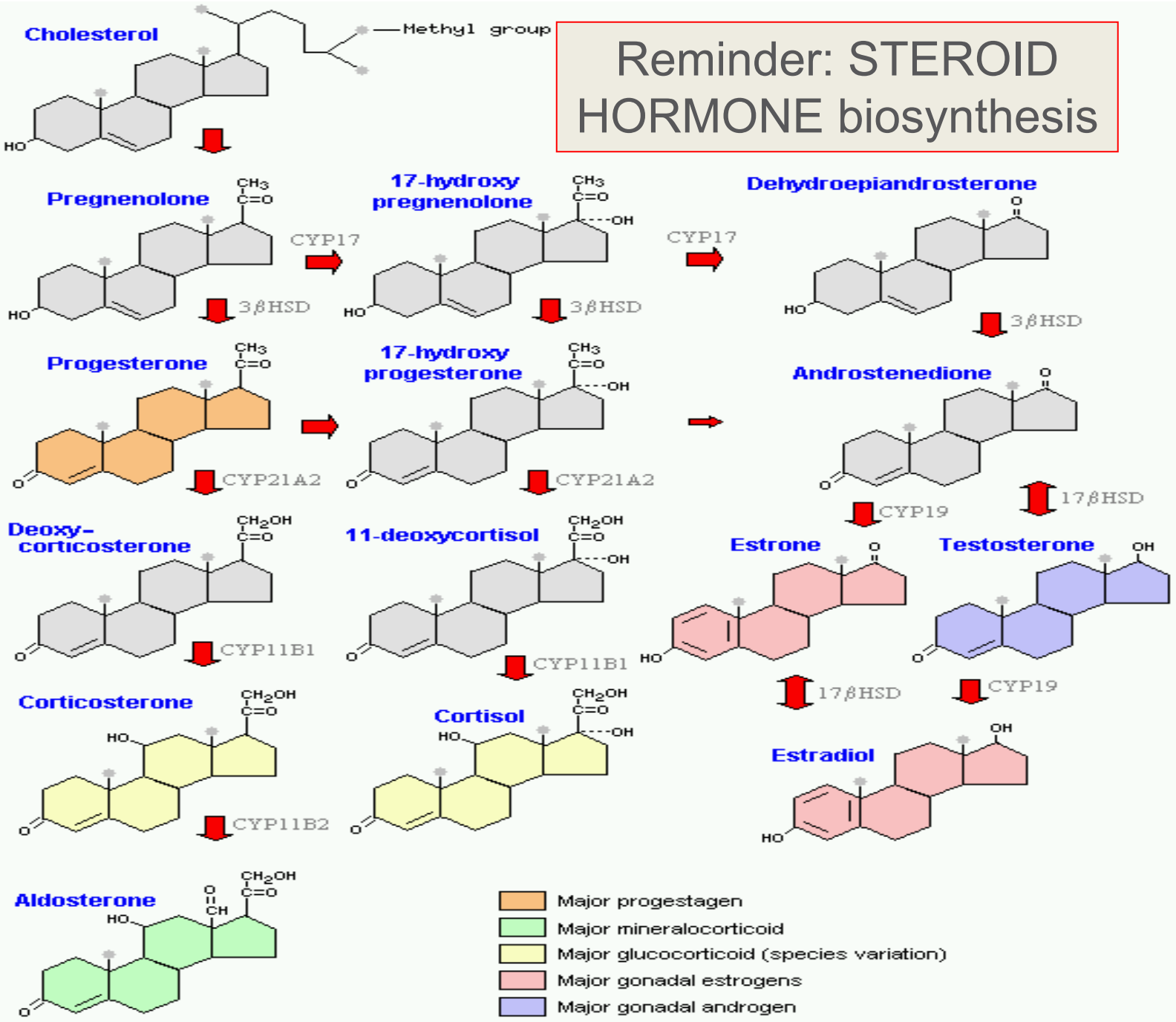


Pregnenolone

Made directly from cholesterol, the precusor molecule for all C_{18}, C_{19} and C_{21} steroids

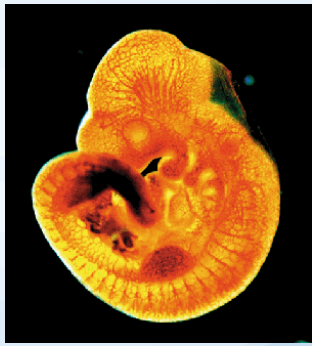


Reminder: STEROID HORMONE biosynthesis



Endocrine disruption

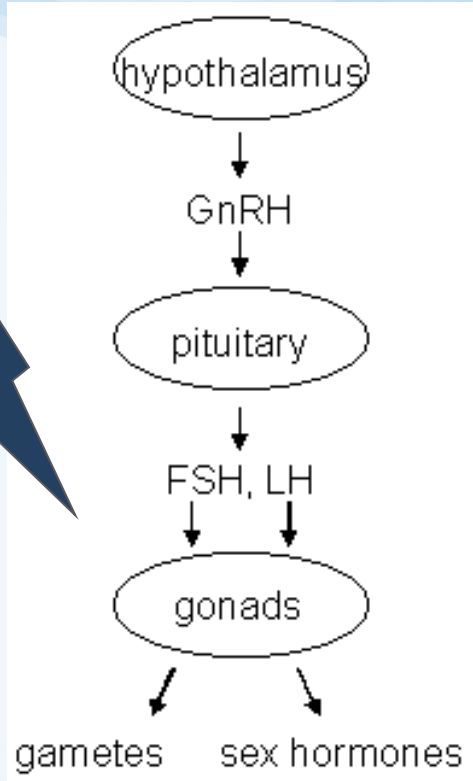
- **Interference of xenobiotics with normal functioning of hormonal system**



Known consequences

→ Disruption of homeostasis, reproduction, development, and/or behavior (and other hormone-controlled processes), such as

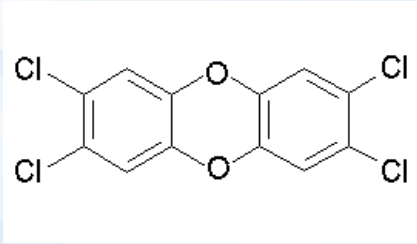
- Shift in sex ratio, defective sexual development
- Low fecundity/fertility
- Hypo-immunity, carcinogenesis
- Malformations
- etc.

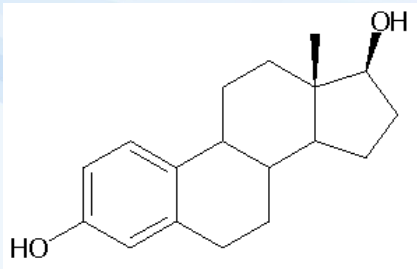


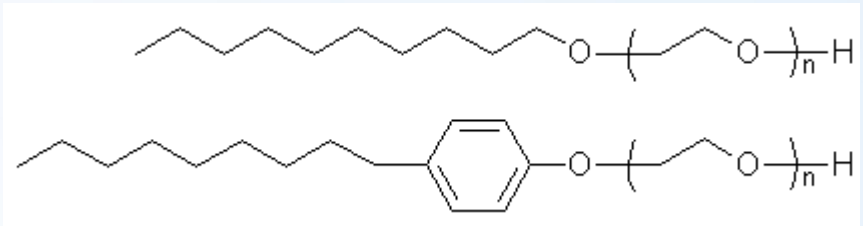
Endocrine disrupters in the environment?

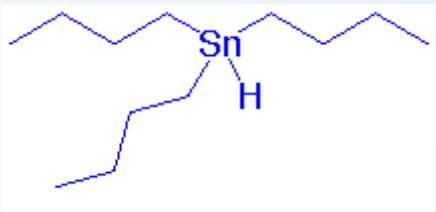
EDCs…

- Persistent Organic Compounds (POPs and their metabolites)
- steroid hormones and their derivatives from contraception pills
- alkylphenols
- organometallics (butyltins)
- pharmaceuticals
- Pesticides
- + number of unknowns …









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Toxicants interact with hormonal system at different levels

Synthesis

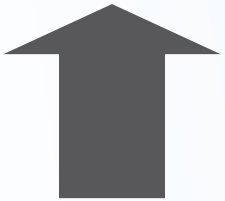
Transport

Interaction with receptors

Metabolization

Consequences (both negative!)

Suppression



Stimulation

Toxicant effects in detail

biosynthesis and release of hormones

e.g. steroidogenesis

e.g. modulation of CYP11A and/or CYP19 activities

binding to plasmatic transport proteins

e.g. down-regulation of receptor levels

binding to nuclear hormonal receptor (HR)

activation of HR
(dissociation of associated heat shock proteins, formation of homodimers)

e.g. modulation of other nuclear receptors
(PPAR/RXR, RXR/TR)

binding of the activated receptor complex to specific DNA motifs - HREs

chromatin rearrangement and transcription of estrogen-inducible genes

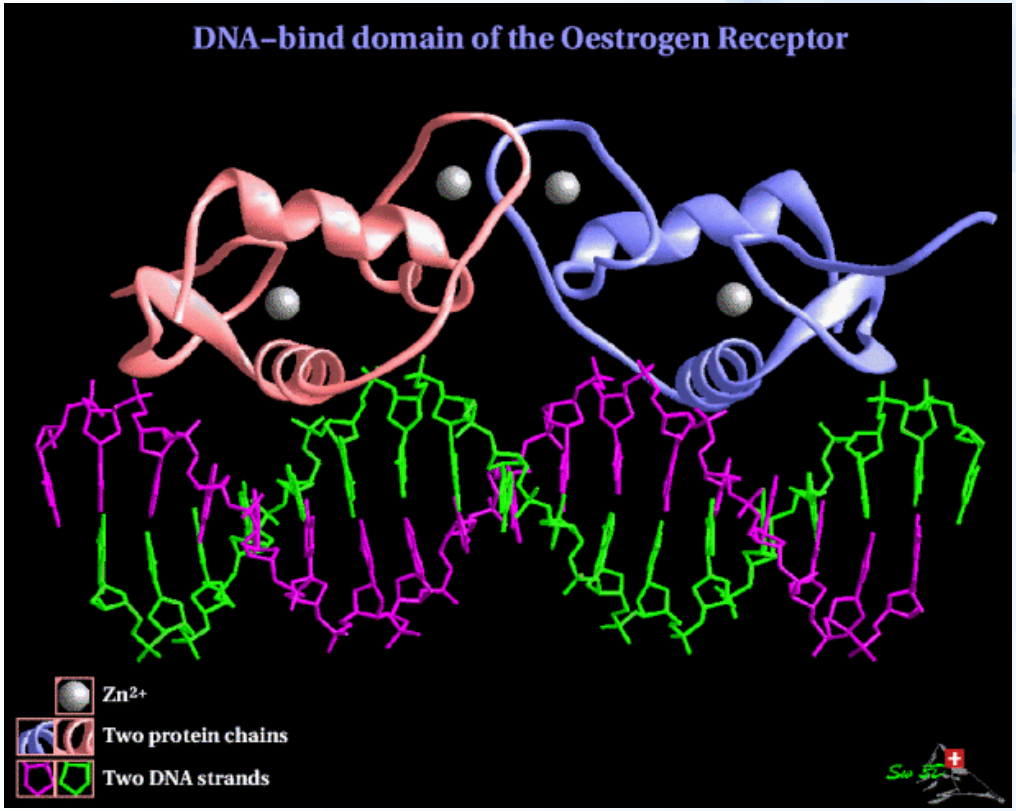
effects at the cellular, tissue, organ, organism, and/or population level

Mechanisms of steroid hormones signalling disruption

- Nonphysiological activation of hormone receptor (HR)
- Binding to HR without activation
- Decrease of HR cellular levels
- Disruption of the „master“ hormones (FSH/LH)
- Changes in hormone metabolism

ESTROGEN RECEPTOR – ER

the most studied target of EDCs



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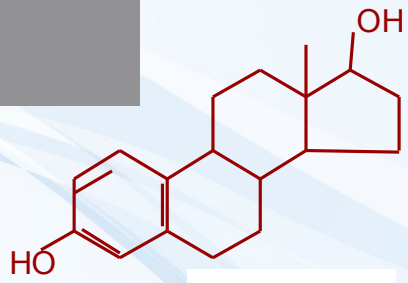
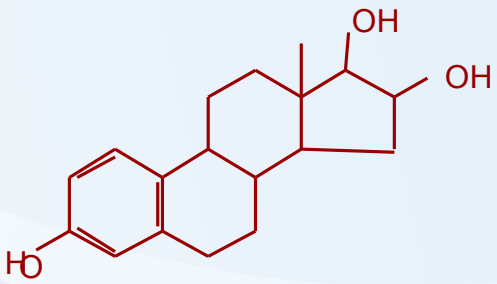
Estrogens



17-β-estradiol



estriol

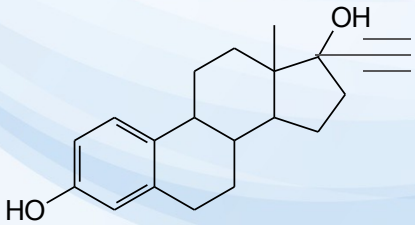
- key roles in female hormone regulation and signalling
- responsible for metabolic, behavioural and morphologic changes occurring during stages of reproduction
- involved in the growth, development and homeostasis in a number of tissues
- control the bone formation, regulation of homeostasis, cardiovascular system and behaviour
- regulate production, transport and concentration of testicular liquid and anabolic activity of androgens in males

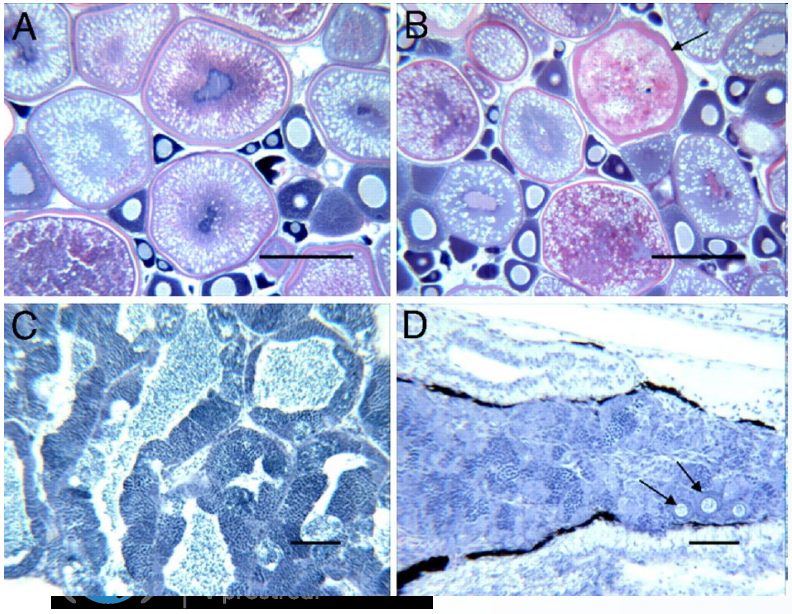
- Synthesis in ovaries

- DISRUPTION → many effects known in aquatic biota & laboratory organisms

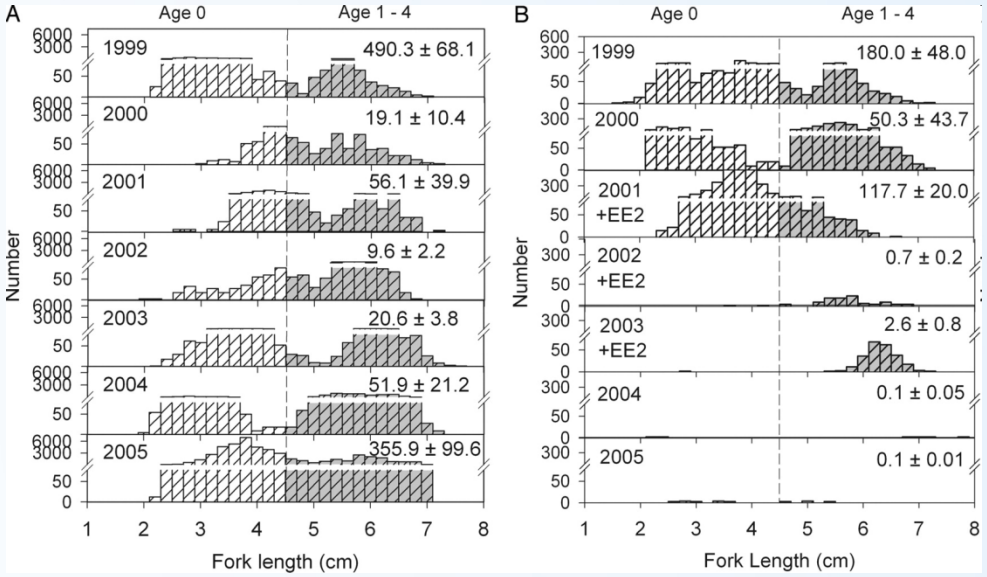
Kidd, K.A. et al. 2007. Collapse of a fish population following exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences* 104(21):8897-8901

5 ng/L (!)
7 years





Controls +Ethinylestradiol

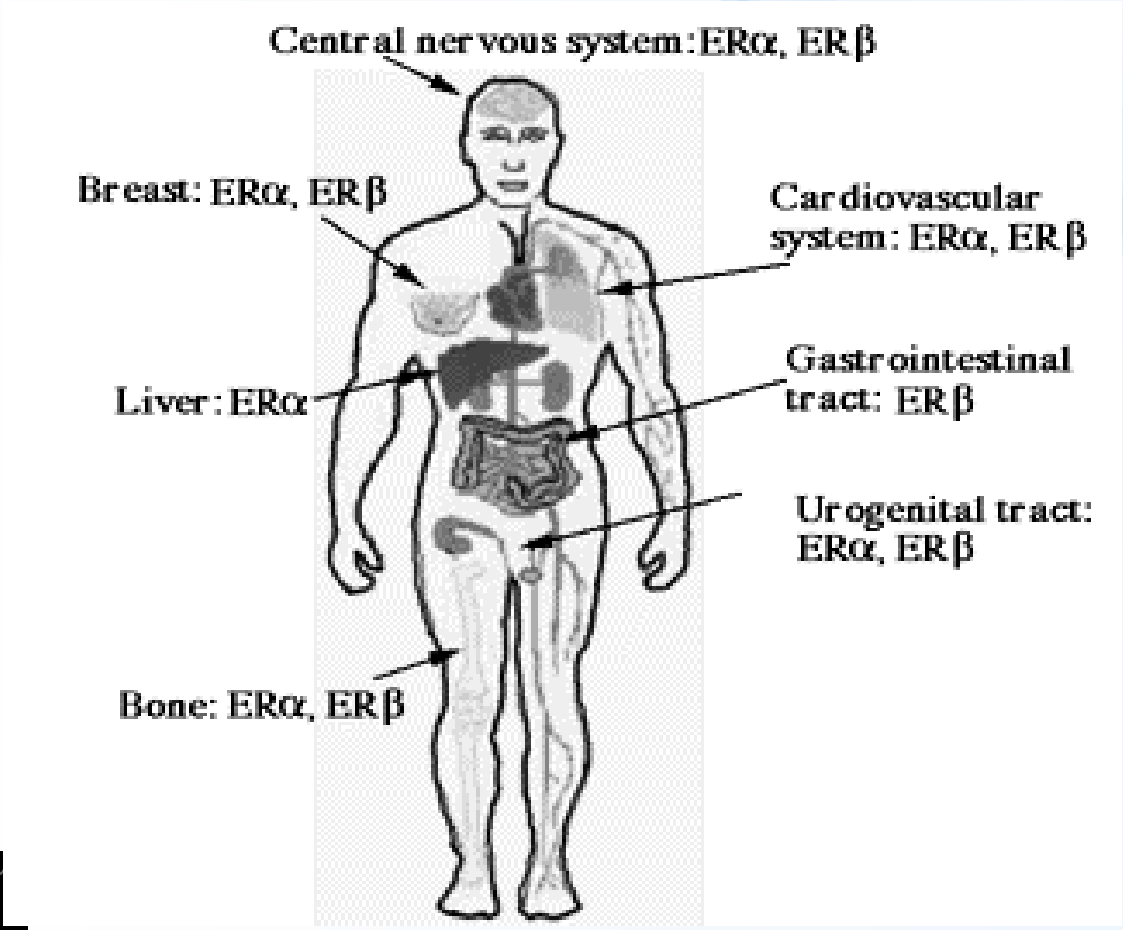


ESTROGEN RECEPTORS- subtypes

ER-α (in breast, ovary, brain, liver, bone and cardiovascular system, adrenals, testis and urogenital tract)

ER-β (in kidneys, prostate and gastrointestinal tract)

(ER-γ in fish)

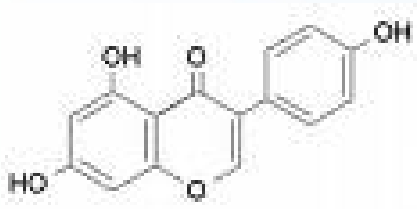


Environmental estrogens (xenoestrogens, exoestrogens)

a diverse group of substances
do not necessarily share structural similarity to the prototypical estrogen 17β-estradiol
may act as AGONISTS **and/or** ANTAGONISTS (depending on situation and concentration!)

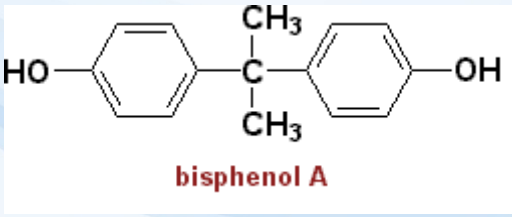
Natural products

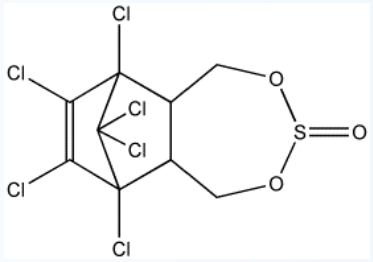
genistein
naringenin
coumestrol
zearalenone

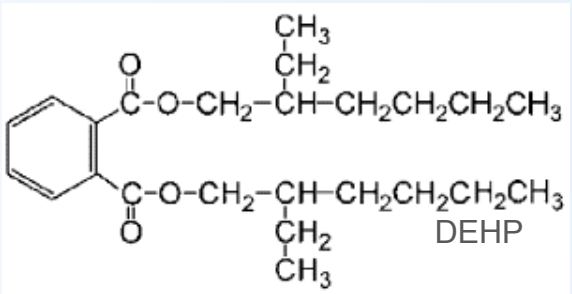


Industrial chemicals

Bisphenol A
Nonionic surfactants
Pthalate esters
endosulfan







Various POPs

DDT
kepone
PCBs/OH-PCBs
PAHs and dioxins

Pharmaceuticals

Ethinyl estradiol
Diethylstilbestrol
gestodene
norgestrel

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Exoestrogens - Relative Potencies to bind to ERa (REPs)

REP – a measure of toxic potency of a compound (similar also at other NRs)

Chemical group	Substance	REP
Endogenous hormones	Estradiol	1
	Estriol	$6{,}3.10^{-3}$
	Testosteron	$9{,}6.10^{-6}$
Phytoestrogens	Cuomestrol	$6{,}8.10^{-3}$
	Genistein	$4{,}9.10^{-4}$
Pesticides	o,p´-DDT	$1{,}1.10^{-6}$
PCBs	2,4,6-trichlorbiphenyl-4´-ol	1.10^{-2}
	2,5-dichlorobiphenyl-4´-ol	$6{,}2.10^{-3}$
	3,3´,5,5´tetrachlorobiphenyl-4,4´-diol	$1{,}6.10^{-4}$
alkylphenoles	4-tert-oktylphenol	$3{,}6.10^{-6}$
phthalates	butylbenzylphthalate	4.10^{-6}

REP (RElative Potencies) of selected compounds related to 17-β-estradiol derived from reporter yeast assay

How to assess for ESTROGENICITY?

number of in vivo and in vitro methods available

Assay (ref.)	Exposure type	Detects ER-dependent agents?	Detects non-ER-dependent agents?	Distinguishes agonist versus antagonist?	Pharmacokinetic and metabolism included?
Receptor-based assays					
Receptor binding assay (27)	Cell lysate	Yes	No	No	No
Receptor activation assay (32–34)	Cells in vitro	Yes	No	Yes[a]	No
In vitro estrogen-regulated response assays					
MCF-7 cell proliferation assay (41)	Cells in vitro	Yes	Limited	Yes[a]	No
Induction assays (46,48)	Cells in vitro	Yes	Limited	Yes[a]	No
DNA synthesis assays (47)	Cells in vitro	Yes	Limited	Yes[a]	No
In vivo estrogen-regulated response assays					
Uterotrophic response assay (49)	Whole animal	Yes	Limited	Yes[a]	Yes
Vaginal cornification assay (50)	Whole animal	Yes	Limited	Yes[a]	Yes
Vaginal opening (11)	Whole animal	Yes	Limited	Yes[a]	Yes
Uterine fluid imbibition (11)	Whole animal	Yes	Limited	Yes[a]	Yes
Uterine epithelial hypertrophy (51)	Whole animal	Yes	Limited	Yes[a]	Yes
Inhibition of steroid synthesis assays					
In vitro ovarian steroid assay (55)	Minced tissue	No	Yes	Yes	No
Ex vivo ovarian steroid assay (56)	Whole animal	No	Yes	Yes	Yes

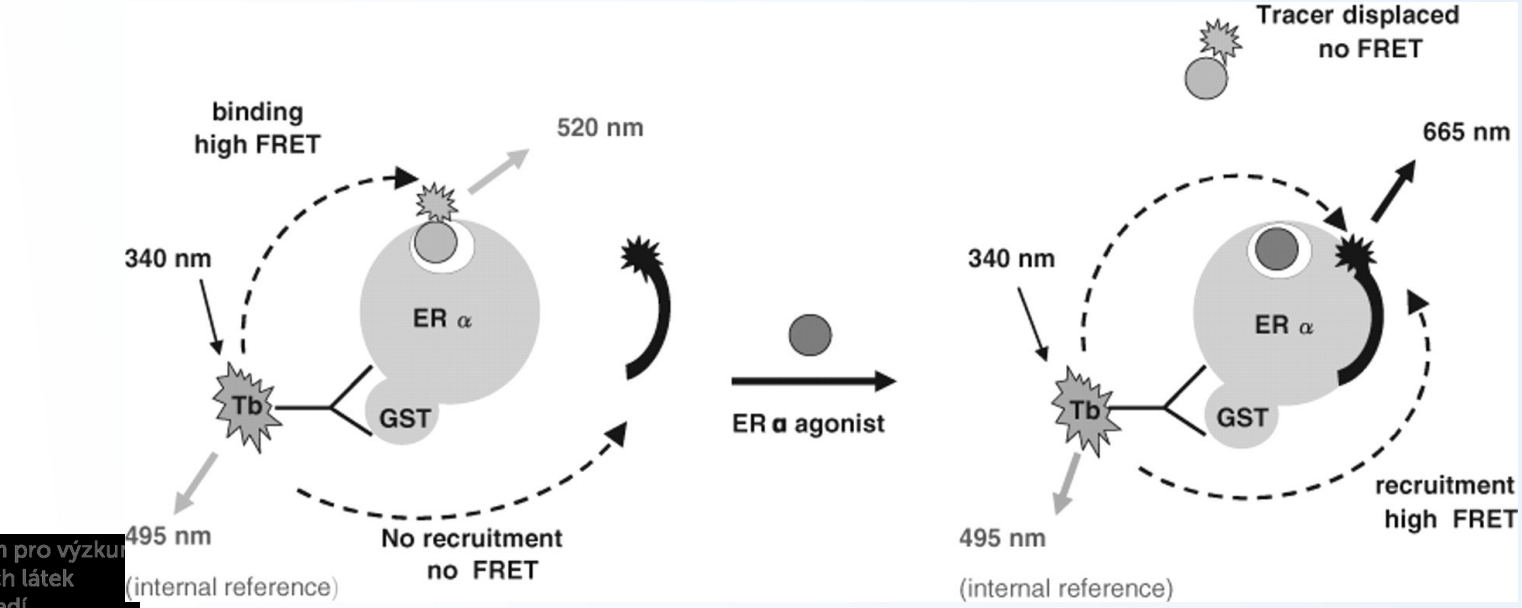
[a]Detection of antagonists requires use of additional groups with test material + estradiol.

Janošek, J., Hilscherová, K., Bláha, L., and Holoubek, I. (2006). Environmental xenobiotics and nuclear receptors-Interactions, effects and in vitro assessment. *Toxicology in Vitro* 20, 18-37.

In vitro assays for estrogenicity

- INTERACTION (BINDING) to the receptor
  - competitive ligand binding assays
    - Various variants (e.g. displacement of radioactive substrate, fluorescence resonance energy transfer (*FRET*) techniques etc.

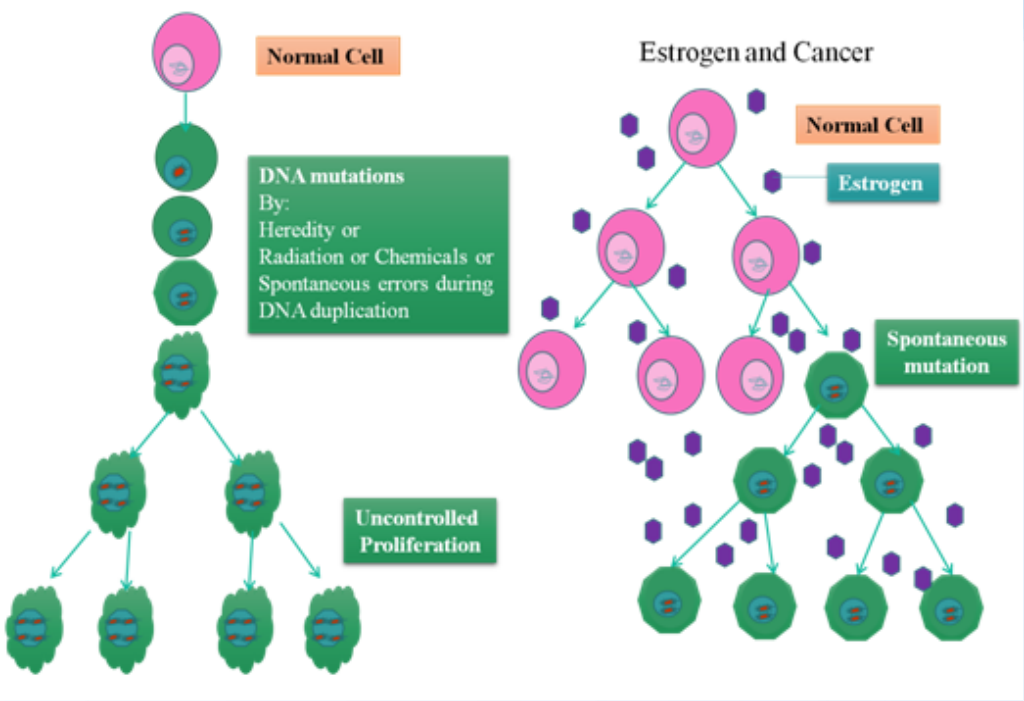
→information only about "binding potency" but the effect remains unknown (? Activation / suppression / no effect ?)



In vitro assays for estrogenicity

- Testing the effect at cellular level
  → interference with receptor biological activity

- **Cell proliferation assays**
  - Estrogens induce proliferation
- **Endogenous protein expression** (or enzyme activity) assays
  - Often reporter gene assays



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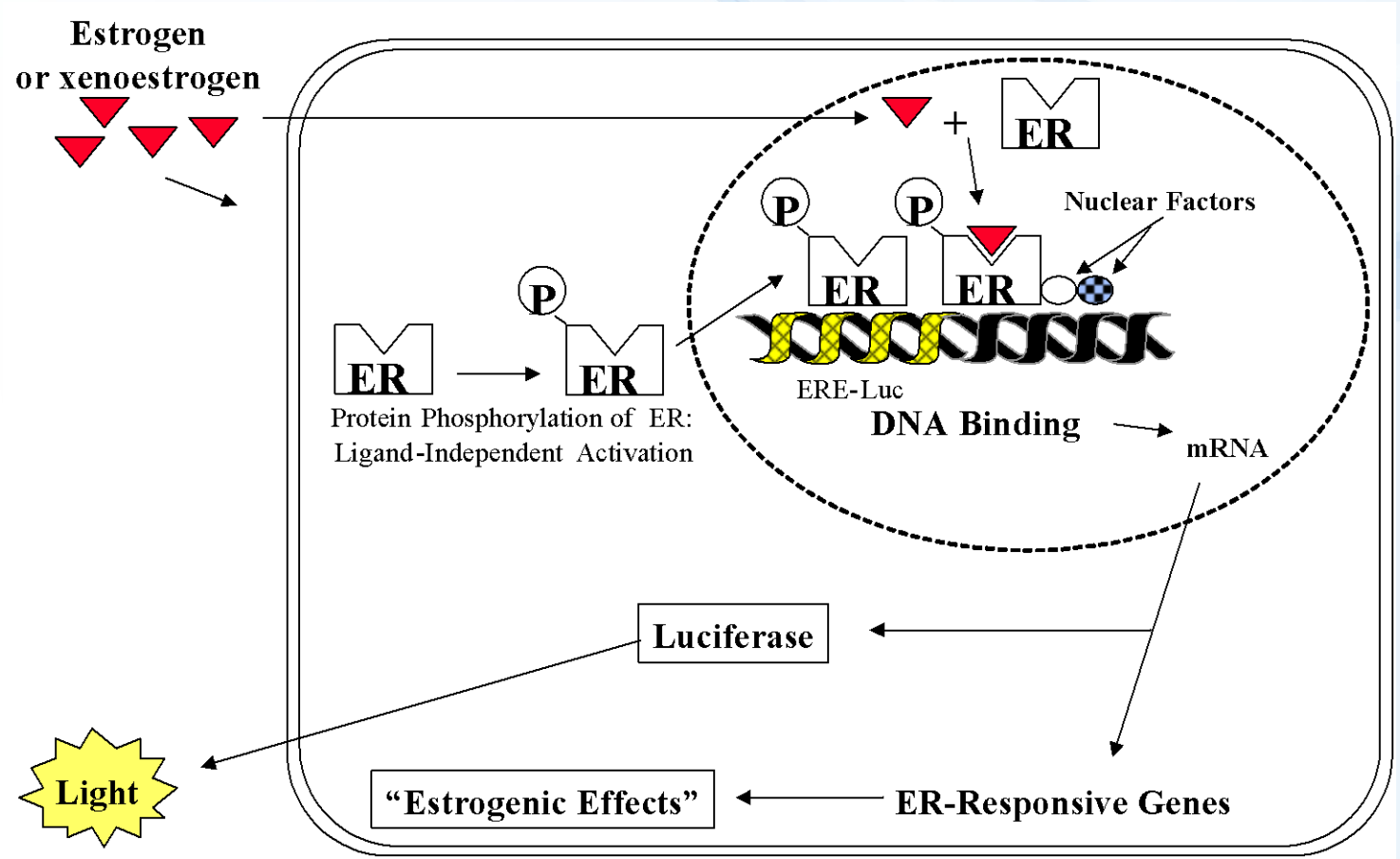
In vitro ER- mediated effects **luciferase reporter assay**

Genetically modified cells (e.g. Breast carcinoma – carrying ERs, stable transfection with firefly luciferase gene)



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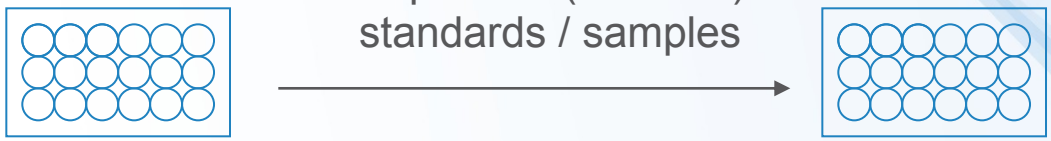
Luciferase reporter assay

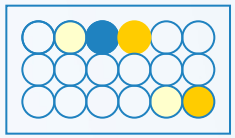


Exposure (6 – 24 h)
standards / samples

96 microwell plate
cultivation of transgenic cell lines

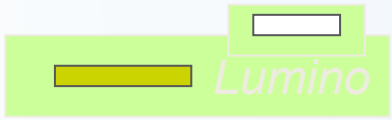
ER: breast carcinoma **MVLN cells**

Cell lysis
-> extraction of induced luciferase



SIMILAR DESIGN FOR OTHER NRs

(discussed below):
AhR (H4IIE.luc cells)
AR (MDA cells)
RAR/RXR (P19 cells)



Luminescence determination
(microplate luminescence reader)

Centrum pro výzkum toxických látek v prostředí

IN VIVO ASSAYS FOR ESTROGENICITY

- uterotropic assay
- vaginal cornification assay

Rat uterus

Control | Estrogen exposure

- production of estrogen-inducible proteins (e.g. **vittelogenin** and zona radiata protein)

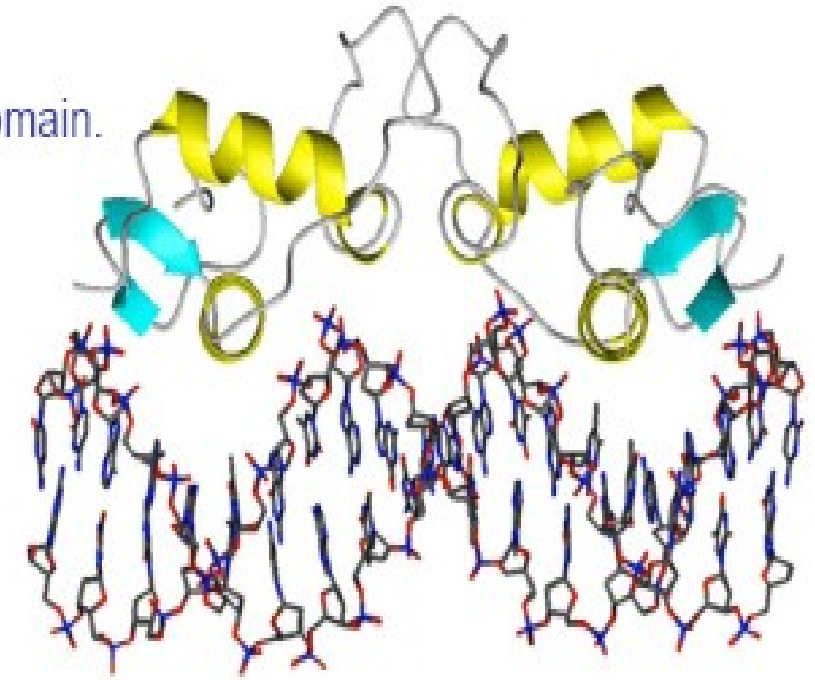
  → also discussed at “biomarkers” part

- standard test procedures for reproductive and developmental toxicity
  - using mice, rats, fish, amphibians etc.

ANDROGEN RECEPTOR (AR)

role in toxicity confirmed ... but less explored than ER

Androgen receptor DNA binding domain.



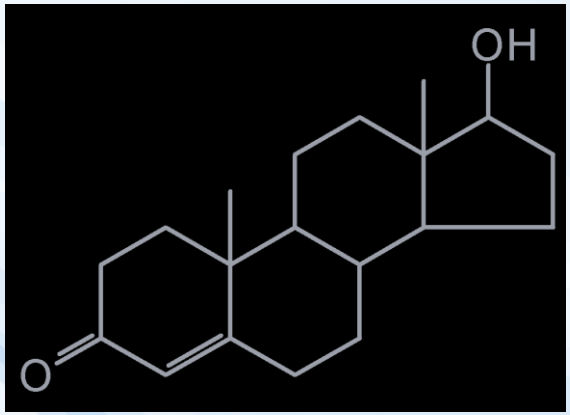
Centrum pro výzkum toxických látek v prostředí

Androgens

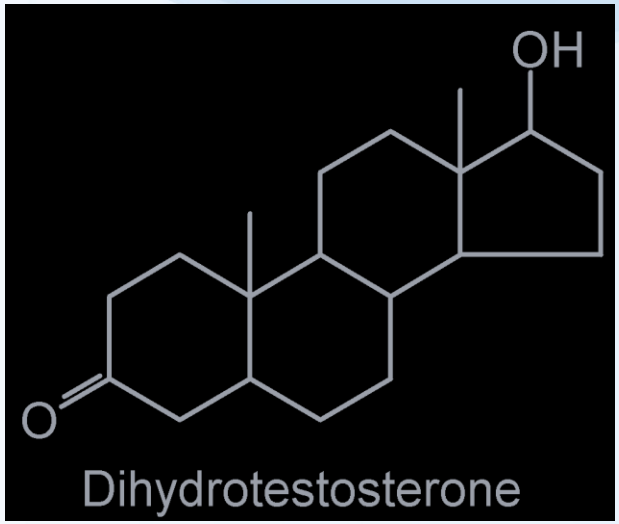
- Role in males similar to the of estrogens in females
  - development of male sexual characteristics
  - stimulating protein synthesis, growth of bones
  - cell differenciation, spermatogenesis
  - male type of behaviour

Androgens

- **Endogenous ligands – androgen hormones**
  - **Two key androgens**
    - **testosterone (T)**
    - **dihydrotestosterone (DHT)**
  - Other androgens – androstanediol, dehydroepiandrosterone, androstenedione



Testosterone



Dihydrotestosterone

- **T: synthesis in testis (Leydig cells)**
  - in lesser extent in adrenals
- **DHT**: Formed **extratesticulary** from T
  - In several tissues (seminal vesicles, prostate, skin) higher affinity to androgen receptor than T
  - Daily production 5-10% of testosterone

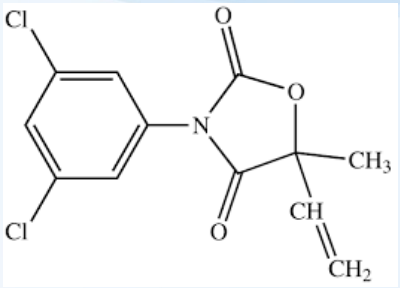
Mechanisms of androgen signalling disruption

1) Binding to AR

- Mostly competitive inhibition
- xenobiotics mostly DO NOT activate AR-dependent transcription

- Only few compounds able to activate AR in the absence of androgen hormones but they are **anti-androgenic in the presence of strong androgens like T or DHT**
  - metabolites of fungicide vinclozoline, some PAHs

vinclozoline



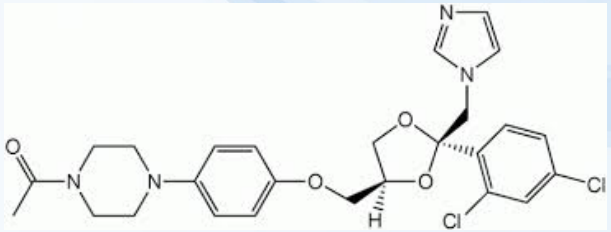
2) FSH/LH (gonadotropins) signalling disruption – less explored

- FSH/LH expression - regulation via negative feedback by testosterone
- Suppression → alterations of spermatogenesis

Mechanisms of androgen signalling disruption

3) Alterations of testosterone synthesis

- Inhibition of P450scc needed for side chain cleavage of cholesterol or inhibitions of 17-beta-hydroxylase and other CYPs
  - fungicide ketoconazol



4) Testosterone metabolic clearance

- Induction of detoxification enzymes (UDP-glucuronosyltransferase or monooxygenases CYP1A, 1B)
  - Pesticides endosulfan, mirex, o-p´-DDT

Effects of **male** exposure to antiandrogens

- **Exposure during prenatal development:**
  - malformations of the reproductive tract
    - reduced anogenital distance
    - **hypospadias** (abnormal position of the urethral opening on the penis)
    - vagina development
    - undescendent ectopic testes
    - atrophy of seminal vesicles and prostate gland

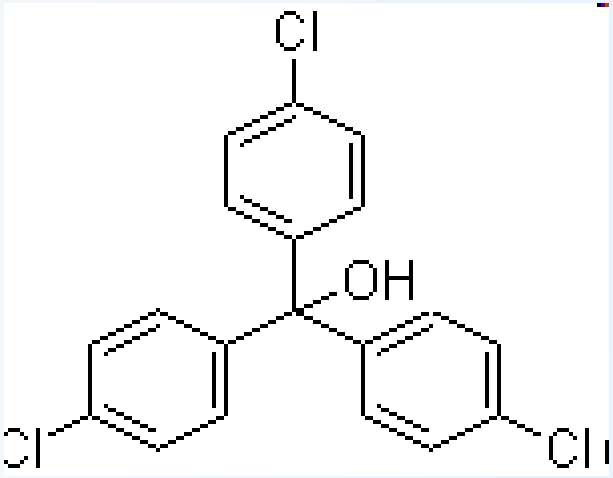
Seek google for illustrations

- **Exposure in prepubertal age:**
  - delayed puberty
  - reduced seminal vesicles
  - reduced prostate

- **Exposure in adult age:**
  - oligospermia
  - azoospermia
  - loss of sexual libido

Antiandrogenic compound

- tris-(4-chlorophenyl)-methanol
  - Ubiquitous contaminant of uncertain origin
  - Probable metabolite of DDT-mixturec
  - Levels in human blood serum cca. 50nM
  - antiAR - EC50 – cca. 200nM



Centrum pro výzkum toxických látek v prostředí

AR-binding – potencies (Reference DHT: EC50 ~ 0.1 uM)

Compound	IC_{50} (μM)
Benz[a]anthracene	3.2
Benzo[a]pyrene	3.9
Dimethylbenz[a]anthracene	10.4
Chrysene	10.3
Dibenzo[a,h]anthracene	activation in range 0.1-10μM
Bisphenol A	5
vinclozolin metabolites	9.7
hydroxyflutamide	5
Aroclor typical values	0.25-1.11
Individual PCBs typical values	64 - 87
tris-(4-chlorophenyl)-methanol	0.2

*(Anti)*androgenicity assessment

- **In vivo Hershberger assay**
  - castrated rats treated with examined substance
  - Endpoint – after 4-7 days – seminal vesicles and ventral prostate weight
- **In vivo measurement of testosterone blood levels**

- **In vitro cell proliferation assays**
  - cell lines with androgen-dependent growth: mammary carcinoma cell lines
  - prostatic carcinoma cell lines

- **Receptor-reporter assays**
  - Gene for luciferase (or GFP) under control of AR
    - AR-CALUX (human breast carcinoma T47D)
    - PALM (human prostatic carcinoma PC-3)
    - CHO515 (Chinese hamster ovary CHO)
  - Yeast transfected cells
    - beta-galactosidase reporter

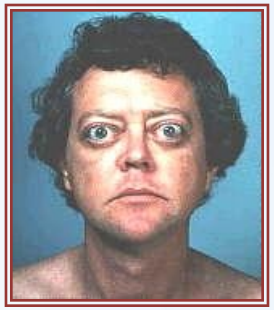
THYROID SIGNALLING

THYROID HORMONES

- Play crucial roles in stimulating metabolism, development and maturation
  - Regulation of metabolism
    - increasing oxygen consumption
    - modulating levels of other hormones (insulin, glucagon, somatotropin, adrenalin)
  - Important in cell differenciation
  - Crucial role in development of CNS, gonads and bones

- EDC compounds interfering with thyroid signalling
  "GOITROGENS"

HYPOTHYROIDISM



HYPERTHYROIDISM

Thyroid hormones

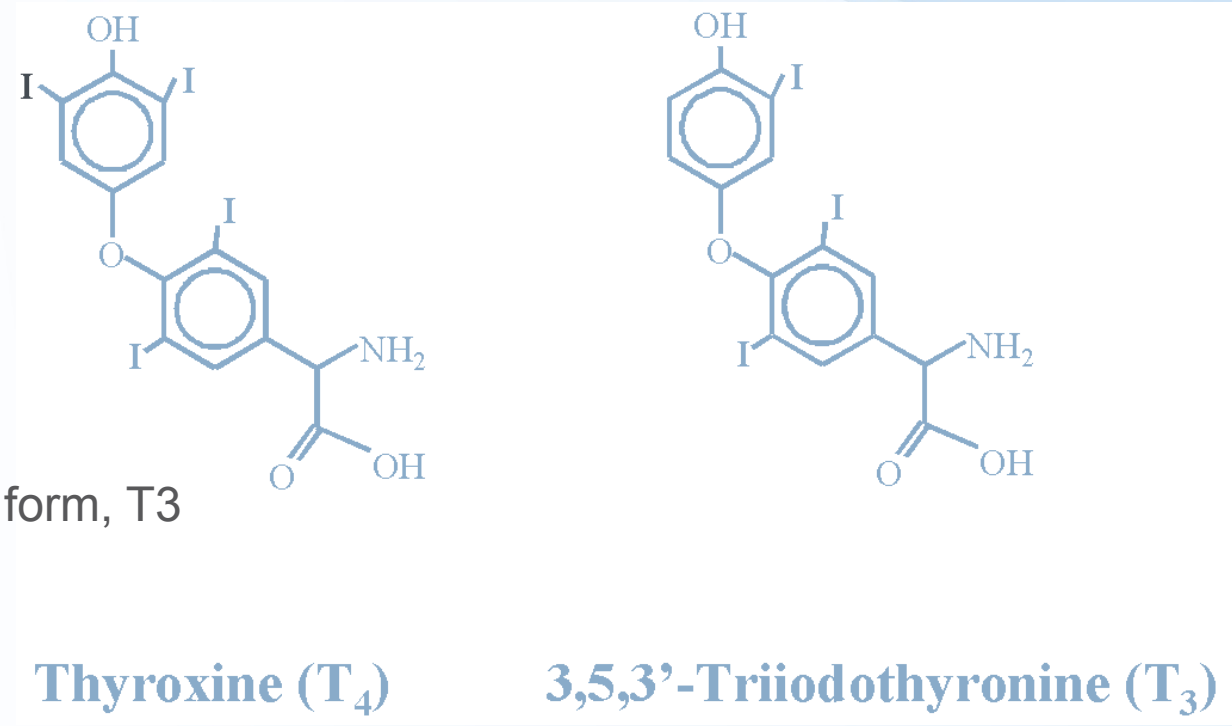
Thyroxine (T4)

Also called tetraiodothyronine
Contains 4 iodide ions

Triiodothyronine (T3)

Contains 3 iodide ions

-Most T3 produced
by deiodination
in target tissues (deiodinases)

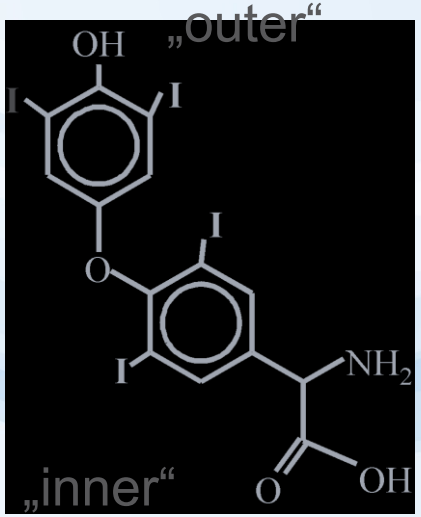


T4 – prohormone
5 -deiodination → active form, T3

Centrum pro výzkum toxických látek v prostředí

Enzymes involved in Thyroid hormone metabolism

- **Thyroid peroxidases**
  - iodination of tyrosyl residues
  - coupling of iodinated tyrosyl residues
- **Thyroid deiodinases**
  - D1, D2 - activation of T4 into T3 via deiodination on „outer“ ring
  - D3 - deactivation into rT3 via deiodination on „inner“ ring



- **Many goitrogens** affect expression, activities and outcomes of these key enzymes
- E.g. Thiocyanate ($[SCN]^-$) or perchlorate ($NaClO_4$)
  →effect on iodine uptake

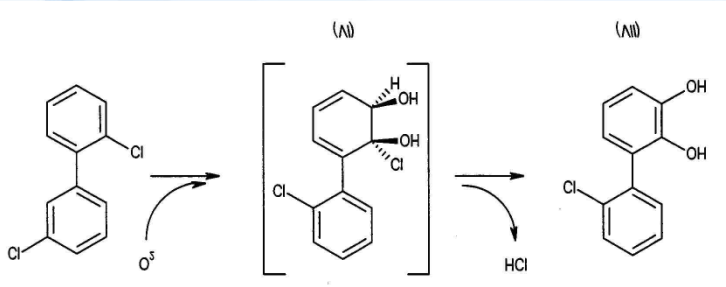
Transport of thyroid hormones in blood

- SPECIFIC TRANSPORTERS in blood
  - regulating free T4 and T3 levels
  - 3 types :
    - Thyroid-binding prealbunin (transthyretin) (20-25%)
    - Albumin (5-10%)
    - Thyroid binding globulin (**TBP**, 75%)

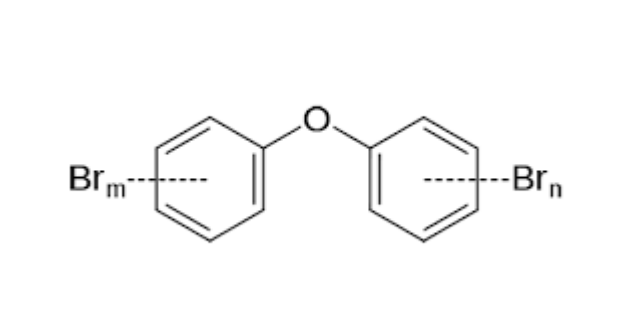
- NUMBER OF EDCs → act on transport proteins
  - OH-PCBs, brominated and chlorinated flame retardants, DDT, dieldrin
  - OH-PCBs – equal affinity to **TBP** as T4 and T3 (!!!)

- Increased levels of "free T4" in blood
  - negative feedback to TSH release
    → increased depletion
    → increased weight,
    histological changes in thyroid gland
  - Documented after exposures to POPs in mammals, birds, fish

Hydroxylated PCB formation



Polybrominated diphenyl ethers (PBDEs) – flame retardants



Centrum pro výzkum toxických látek v prostředí

Other mechanisms of goitrogens' toxicity

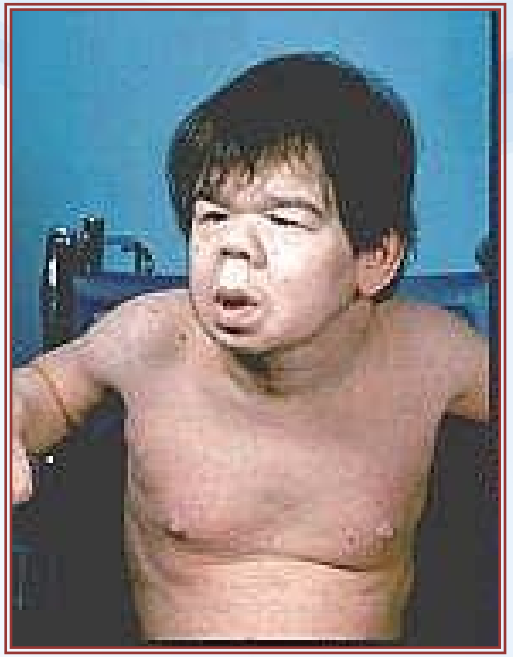
- Competitive binding to TR
  - Probably less important than binding to TBP
    - Chemicals that affect thyroid signalling in vivo mostly don´t bind to TR (DDT, PCBs) or bind with much lesser affinity than T3 (OH-PCBs – 10000x)

- Accelerated depletion of hormones
  - UDP-glucuronosyltransferase – detoxification enzyme (II.biotransformation phase)
  - Induced by PCBs and dioxins
    → indirect goitrogens

Effects of thyroid disruption

- **Exposures during prenatal stages**
  - severe damage of CNS (cretenism, delayed eye opening, cognition)
  - Megalotestis
  - Histological changes in thyroid gland (goitre)

- **Exposures during development**
  - nervous system fails to develop normally
  - mental retardation
  - skeletal development



Assessment of goitrogen effects

(For information only)

- **In vivo approaches**
  - TH serum levels – simple, nondestructive x variation within time of day, age, sensitive to other than biochemical stresses
  - Thyroid gland weight and folicular cells number
  - Developmental toxicity assays - delayed eye opening, abnormalities in brain development and cognition, increased testis weight and sperm counts
  - Perchlorate discharge test (TH synthesis)
  - Hepatic UDP-glucuronosyltransferase activity (marker of enhanced TH clearance from serum)

- **In vitro**
  - Enzyme inhibition assays (thyroid peroxidase, deiodinases) – assessment of thyroid metabolism
  - Competitive binding assays with TBP
  - TH- dependent proliferation assay (pituitary tumor GH3, thyroid tumors like FRTL-5 cell line) or TSH-dependent proliferation assay (thyroid tumors)
  - Receptor-reporter gene assays with luciferase (monkey kidney CV-1, chinese hamster ovary CHO or insect Sf9 cell lines)

Vitamin A and its derivatives
RETINOIDS

(role in toxicity - still in the research phase)

Centrum pro výzkum
toxických látek
v prostředí

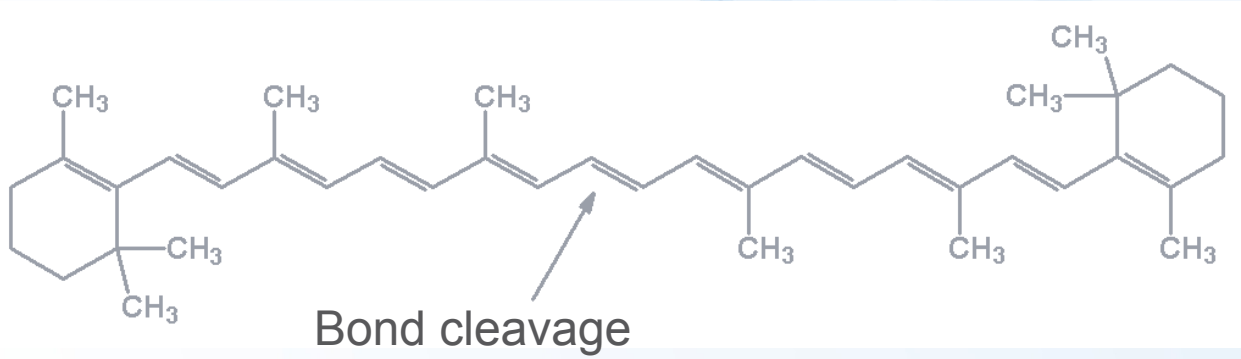
RETINOIDS

Sources: from diet - dietary hormones

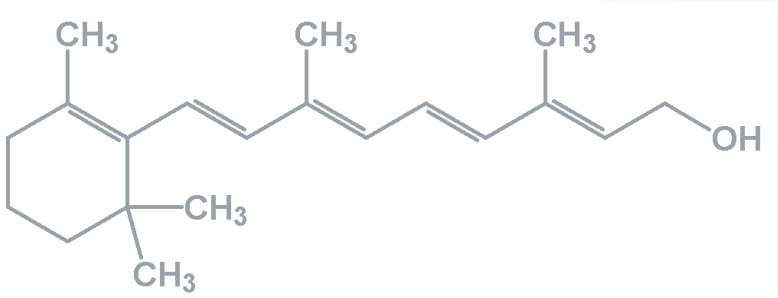
Retinyl esters – animal sources

Plant carotenoids

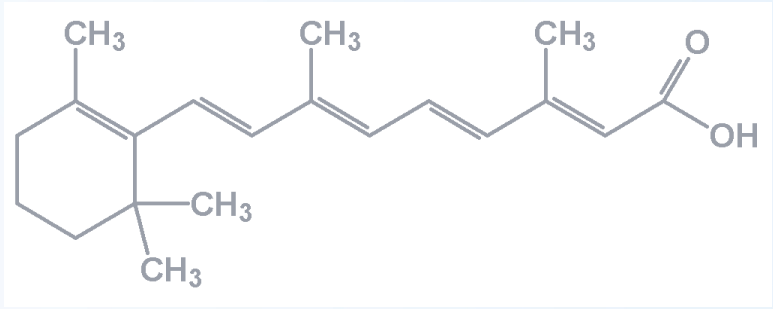
β-karoten



Retinol (vitamin A)



Retinoic Acid



Centrum pro výzkum toxických látek v prostředí

Retinoids and their functions

- Regulation of development and homeostasis in tissues of vertebrates and invertebrates
- Development of embryonic, epithelial cells (gastrointestinal tract, skin, bones)
- Necessary for vision
- Suppressive effects in cancer development
- Important for cell growth, apoptosis and differenciation
- Antioxidative agent
- Affect nervous and immune function

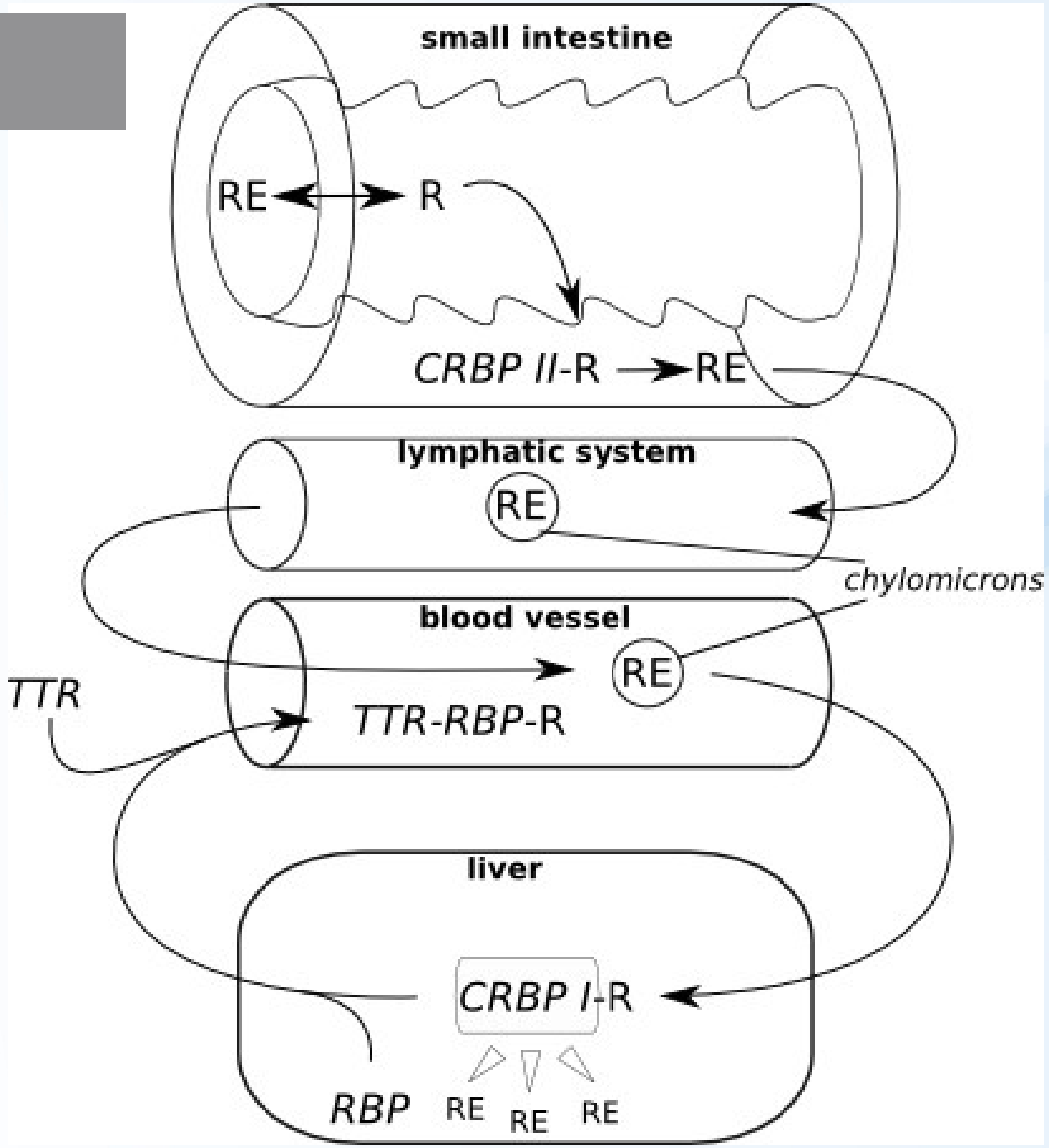
Retinoid transport

RE: Retinol-Ester

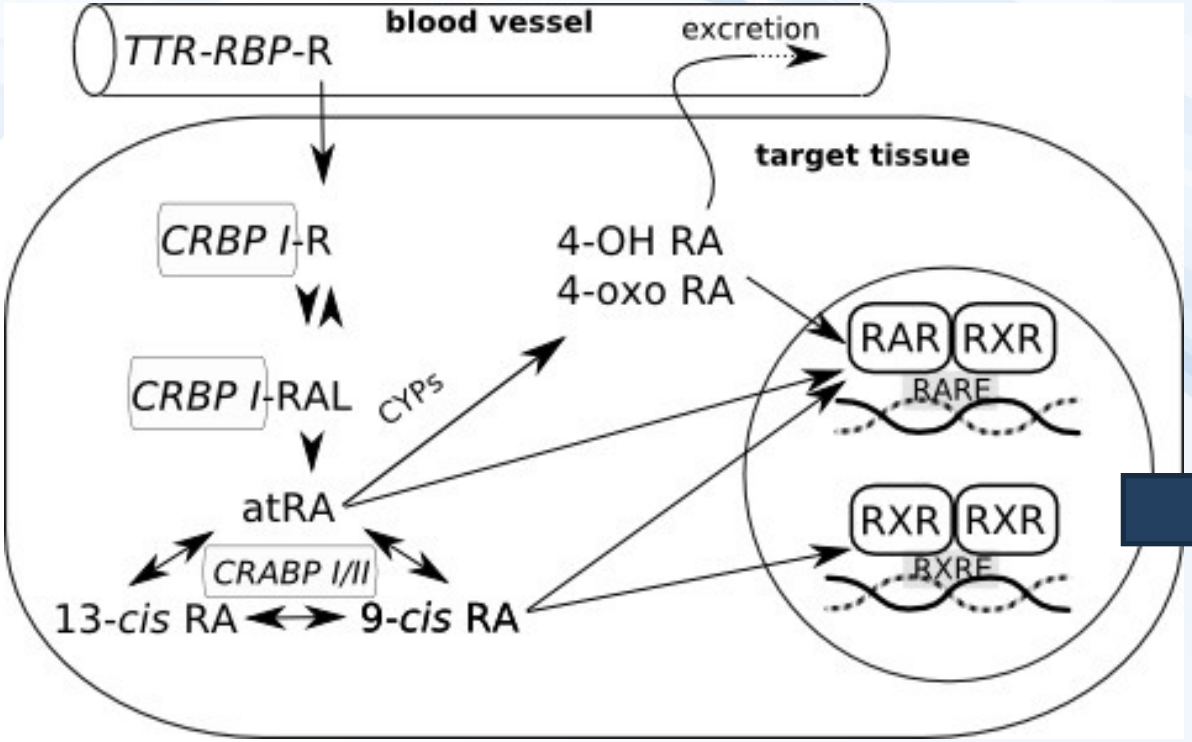
R: Retinol

RBP: Retinol Binding Protein (*LMW*)

TTR: Transthyrethin (*HMW*)



Retinoid fate in the cells



Gene expression

Retinoid binding proteins

CRBP – cellular retinol binding protein
- binding of retinol, immediate decrease of retinol concentration

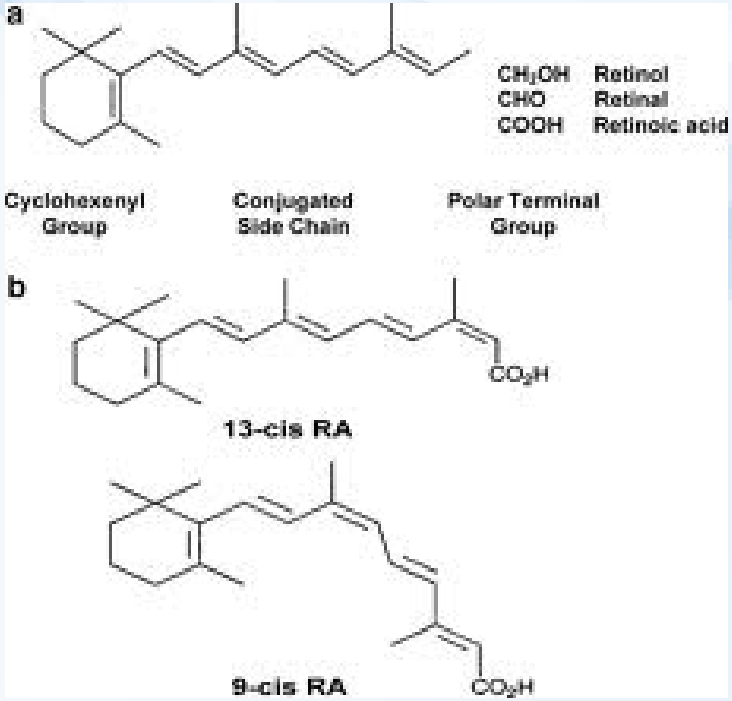
CRBAP – cellular retinoic acid binding protein
- Controlling the ratio free retinol/free retinoic acid

RAR/RXR and RA

- Isoforms of RAR a RXR
  - Formation of homo- and heterodimers
  - 48 possible RAR-RXR heterodimers

  → sensitive regulation of gene expression
- RXR – heterodimers with other receptors
  - VDR, TR, PPAR ... → see crosstalk

- **RETINOIC ACID (RA)**
- 3 basic subtypes
  - all-trans- (ATRA)
  - 9-cis- and 13-cis-retinoic acid
- All-trans RA (ATRA) binds selectively to RAR
- Cis RA bind to both receptor types



Centrum pro výzkum toxických látek v prostředí

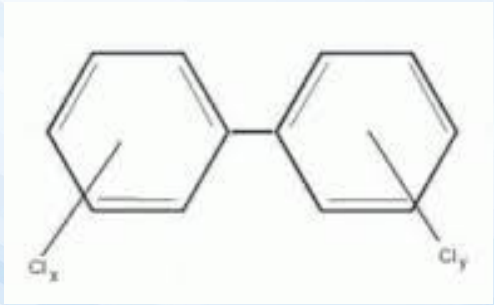
Disruption of retinoid signalling by xenobiotics

- **Relatively little known - possible modes of action:**
  - Metabolization of retinoids by detoxication enzymes
  - Disruption of binding retinoids to retinoid binding proteins
  - Retinoids as antioxidants may be consumed cause of oxidative stress caused by xenobiotics
  - Interference of chemicals (binding to RAR/RXR)

- **Decreased retinoid levels in organisms**
  - Downregulation of growth factors
  - Xerophtalmia, night blindness
  - Embryotoxicity, developmental abnormalities
- **Increased ATRA concentration**
  - teratogenic effects

Disruption of retinoid signalling by xenobiotics

- **Polluted areas**
  - **mostly decrease of retinoid levels**
    - Documented in aquatic birds, mammals and fish



- **Disruption of retinoid transport:** PCBs

- **Effects on retinoid receptors:**
  - RAR, RXR binding and/or transactivation
    - pesticides (chlordane, dieldrin, methoprene, tributyltin…)
    - Effect on ATRA mediated response – TCDD, PAHs

- **Disruption of retinoid metabolism:**
  - **PCDD/Fs,** PAHs, PCBs, pesticides
  - changes of serum concentrations of retinol and RA
  - mobilization of hepatic storage forms

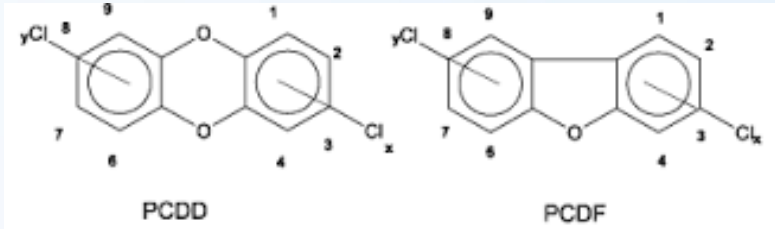


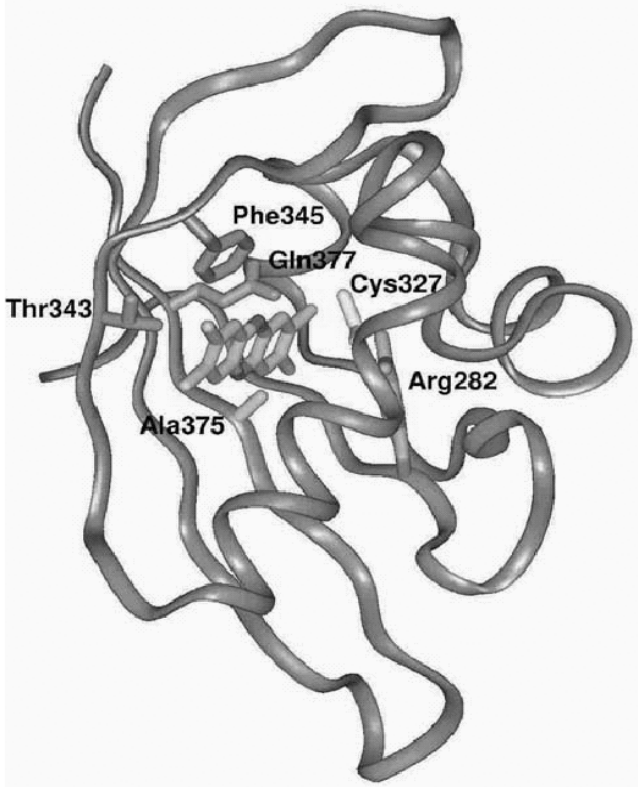
Figure 1. General molecular structure of polychlorinated dibenzo-p-dioxin (PCDD) and dibenzofurans (PCDF)

Centrum pro výzkum toxických látek v prostředí

AhR (Arylhydrocarbon receptor)

AhR structure

Denison et al., Chem Biol. Interact. 141: 3



Centrum pro výzkum toxických látek v prostředí

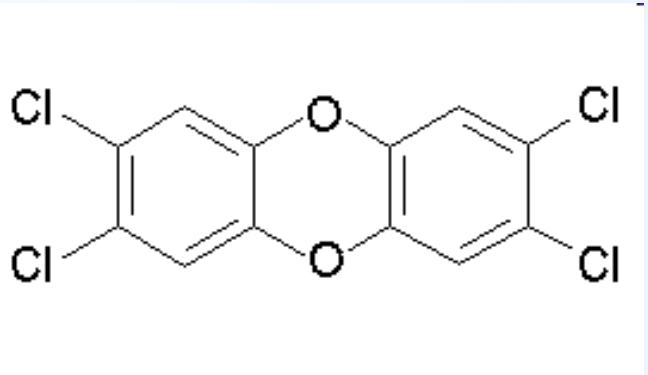
AhR

- ligand-activated transcription factor
  - Similar to all NRs
- activation of many different genes

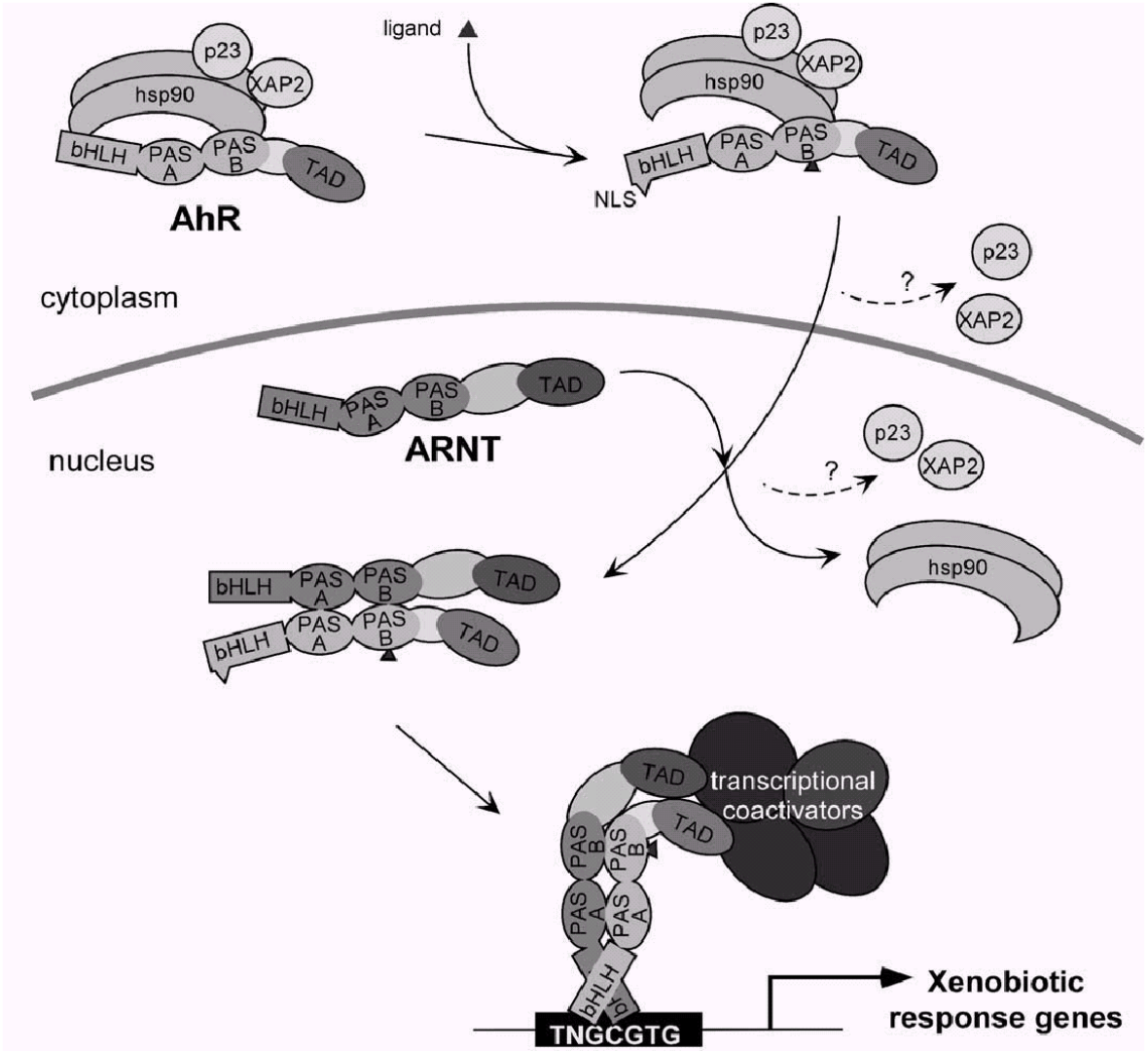
- important mediator of toxicity of POPs – primary target of coplanar aromatic substances
- regulator of xenobiotic metabolism and activation of promutagens

- crossactivation/crosstalk with other NRs

- strongest known ligand - TCDD
  - (not endogeneous !)



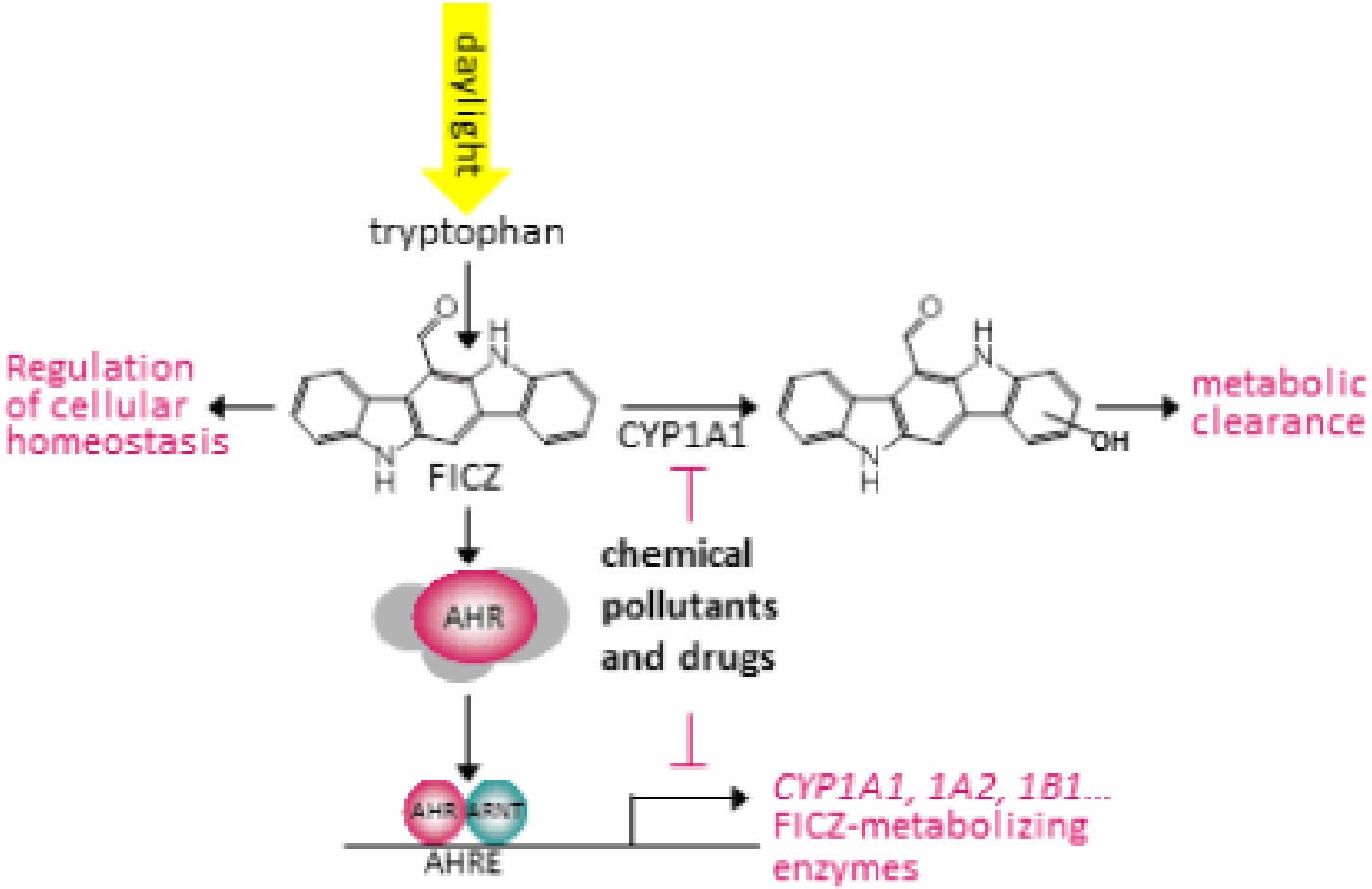
AhR activation



AhR regulated genes

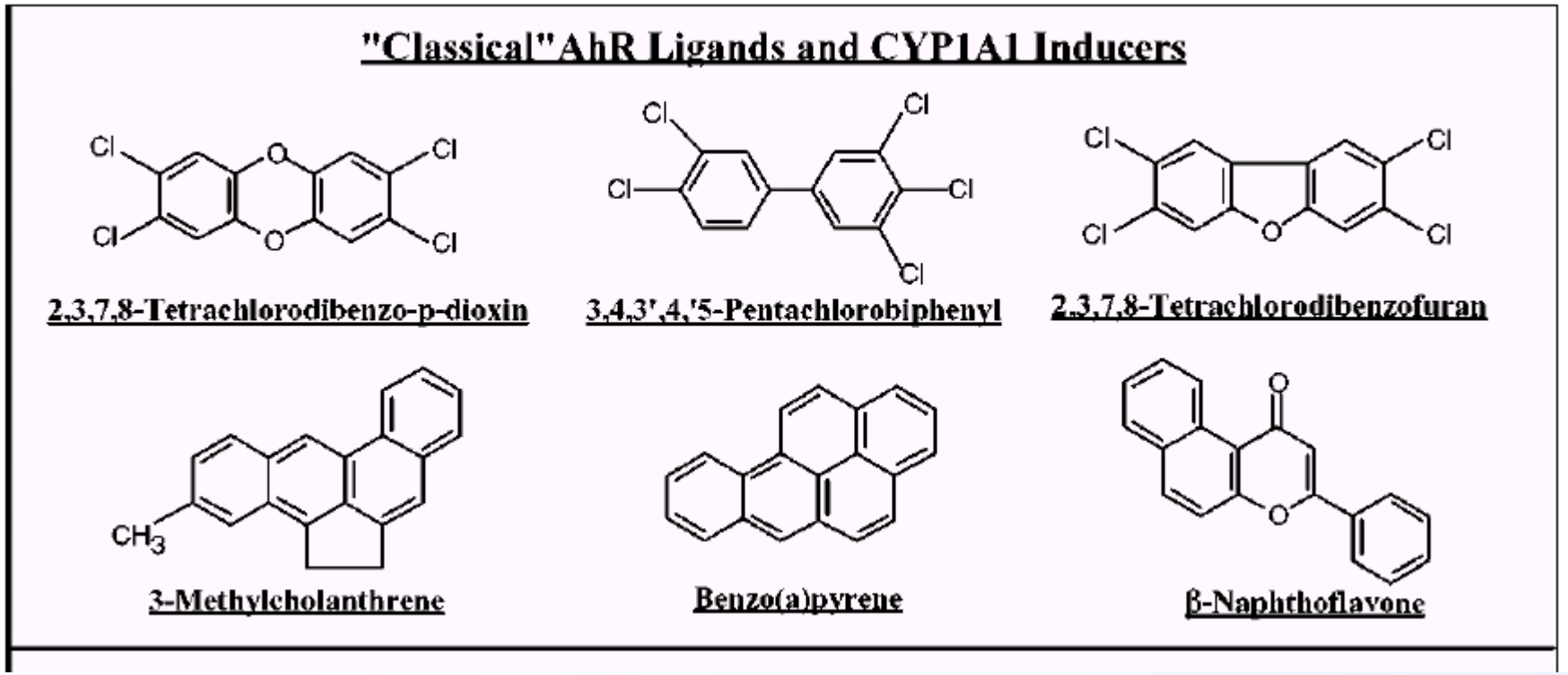
- Many genes contain xenobiotic response elements (XRE) or dioxin responsive elements (DRE) in their promoter region:
  - phase I enzymes - CYP 1A1, CYP 1A2, CYP 1B1;
  - phase II enzymes - UDP-glucuronosyltransferase, GST-Ya, NADP(H):oxidoreductase;
  - other genes - regulation of cell cycle and apoptosis
    - Bax (apoptosis), p27Kip1, Jun B (MAP-kinase), TGF-b

6-formylindolo[3,2-b]carbazole (FICZ)
potent endogenous physiological ligand of AhR



Classical and “non-classical” AhR ligands

Classical = planar structures → direct binding to AhR

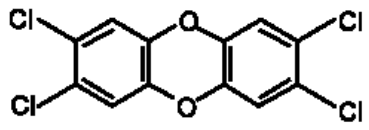


Denison & Nagy, Annu. Rev. Pharmacol. Toxicol. 43:309

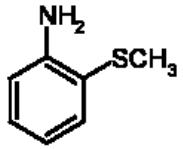
„Non-classical“ AhR ligands – various structures

M.S. Denison et al. / Chemico-Biological Interactions 141 (2002) 3–24

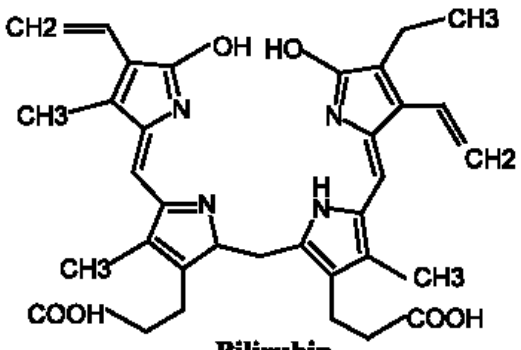
“Classical” ligand



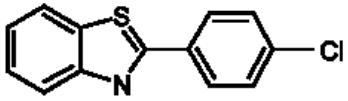
2,3,7,8-Tetrachlorodibenzo-p-dioxin



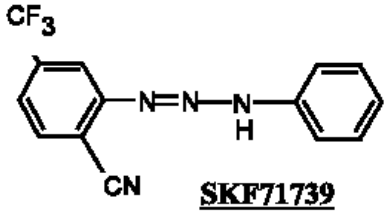
2-(Methylmercapto)aniline



Bilirubin



2-(4'-Chlorophenyl)benzothiazole



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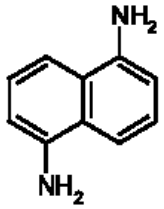
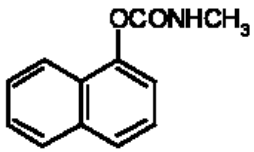
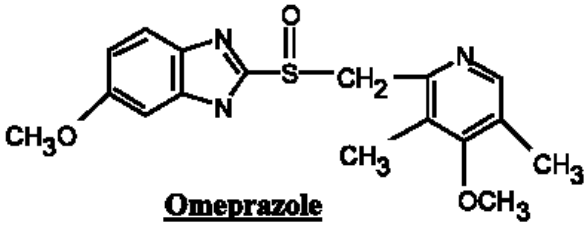


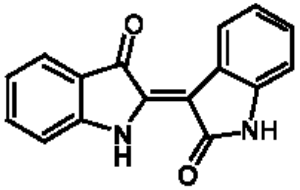
1,5-Diaminonaphthalene



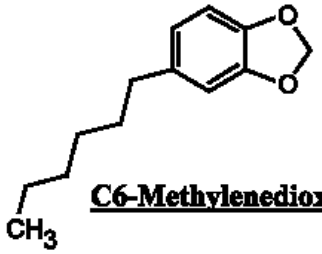
Carbaryl



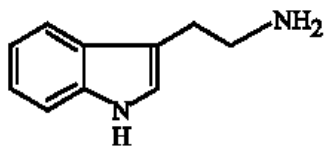
Omeprazole



Indirubin



C6-Methylenedioxybenzene



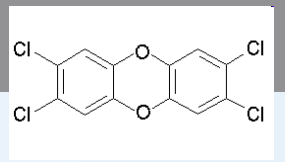
Tryptamine

Physiological role of AhR

- Physiological role for AhR still not known (?)
  - Most likely – “protection” against toxicants → induction of detoxification

- Many adverse effects in **AhR-deficient** mice:
  - significant growth retardation;
  - defective development of liver and immune system;
  - retinoid accumulation in liver;
  - abnormal kidney and hepatic vascular structures.
  - resistant to BaP-induced carcinogenesis and TCDD-induced teratogenesis;
  - no inducible expression of CYP 1A1 and 2.

Biological responses to TCDD



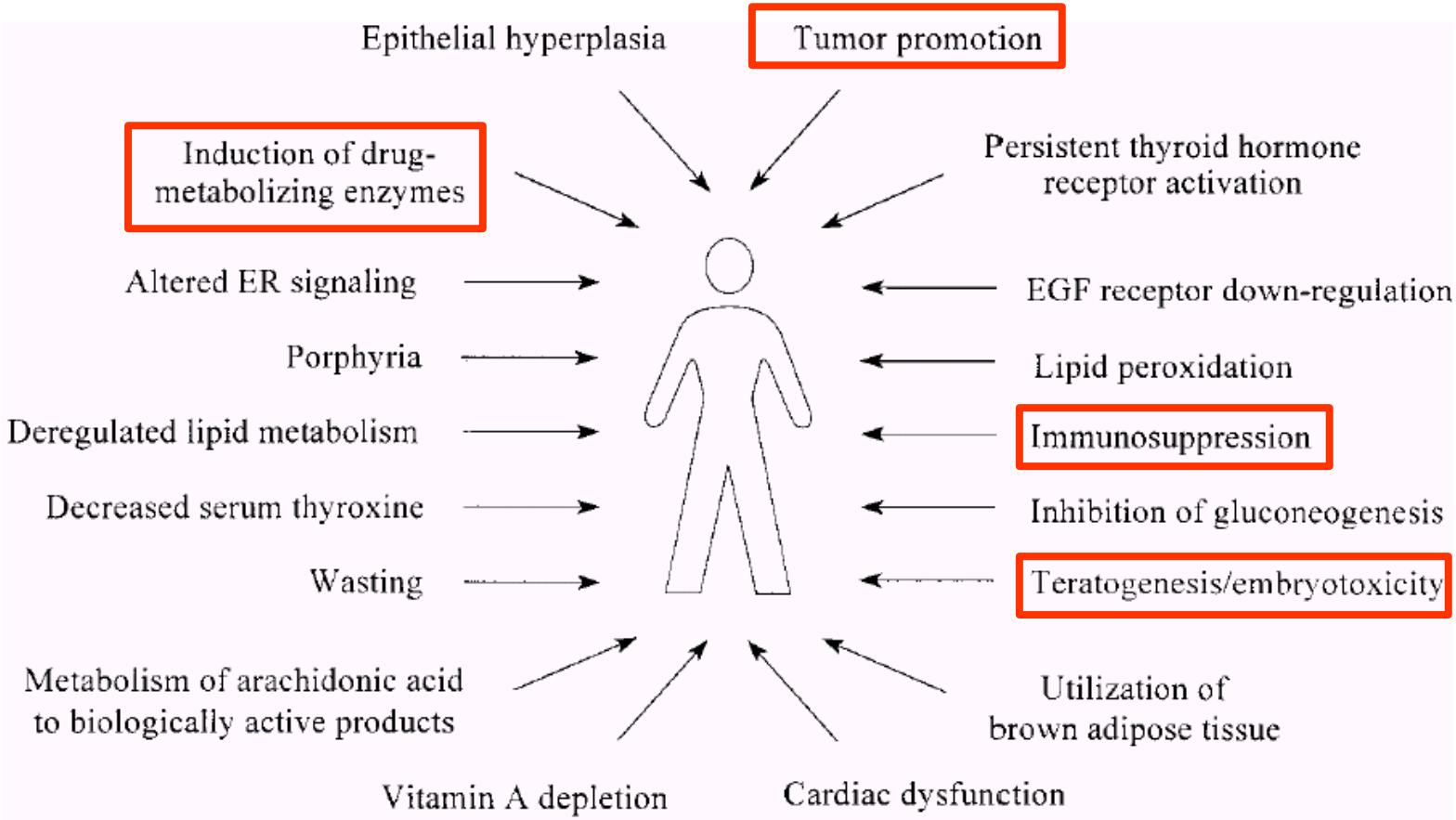


Figure 1 Biological responses to TCDD. A wide variety of cellular processes have been shown to be affected by TCDD.

Schmidt & Bradfield, Annu. Rev. Cell Dev. Biol. 12:55

Toxic equivalency factors (TEF)/TEQ concept

- Toxicity of compounds with similar toxicological properties as TCDD (activating AhR) may be evaluated by TEF/TEQ concept
  - TEF = Toxic Equivalency Factor ("characteristic" of the Chemical)
  - TEQ = Toxic Equivalent (sum of TEFs x concentrations)

- **TEFs are consensus values based on REPs (relative potencies)** across multiple species and/or endpoints.
  - TEFs are based upon a number of endpoints, from chronic in vivo toxicity to in vitro toxicity with the former having the greatest importance in determining overall TEF.

- **TEQs provide a simple**, single number that is indicative of **overall toxicity of a sample** (water, sediment, food) containing a mixture of dioxins and dioxin-like compounds.

- The total potency of a mixture can be expressed in TCDD TEQ concentration
  - i.e. TEQ = concentration corresponding to the effect that would be induced by TCDD

$$\mathrm{TEQ} = \Sigma\{\mathrm{compound}_1 \times \mathrm{TEF}_1 + \ldots$$

$$+ \mathrm{compound}_n \times \mathrm{TEF}_n\}$$

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Toxic equivalency factors for PCDDs, PCDFs and PCBs:

Table 4. Toxic Equivalent Factors established by the WHO (WHO-TEFs) for dioxins and dioxin-like PCBs [4]

PCDD Congener	WHO-TEF	PCDF Congener	WHO-TEF	PCB Congener	WHO-TEF
2,3,7,8-TCDD	1	2,3,7,8-TCDF	0.1	*Non-ortho*	
12,3,7,8-PeCDD	1	12,3,7,8-PeCDF	0.05	PCB#81	0.0005
123478-HxCDD	0.1	23478-PeCDF	0.5	PCB#77	0.0005
123678-HxCDD	0.1	123478-HxCDF	0.01	PCB#126	0.1
12,3,7,89-HxCDD	0.1	123678-HxCDF	0.1	PCB#169	0.01
1234678-HpCDD	0.01	234678-HxCDF	0.1	*Mono-ortho*	
OCDD	0.0001	12,3,7,89-HxCDF	0.1	PCB#105	0.0001
		1234678-HpCDF	0.01	PCB#114	0.0005
		1234789-HpCDF	0.01	PCB#118	0.0001
		OCDF	0.0001	PCB#123	0.0001
				PCB#156	0.0005
				PCB#157	0.0005
				PCB#167	0.00001
				PCB#189	0.0001

Eljarrat & Barceló, Trends Anal. Chem.22: 655

Final concentration is expressed as „Equivalents of TCDD“
(e.g. ng TEQ / kg = ng TCDD / kg)

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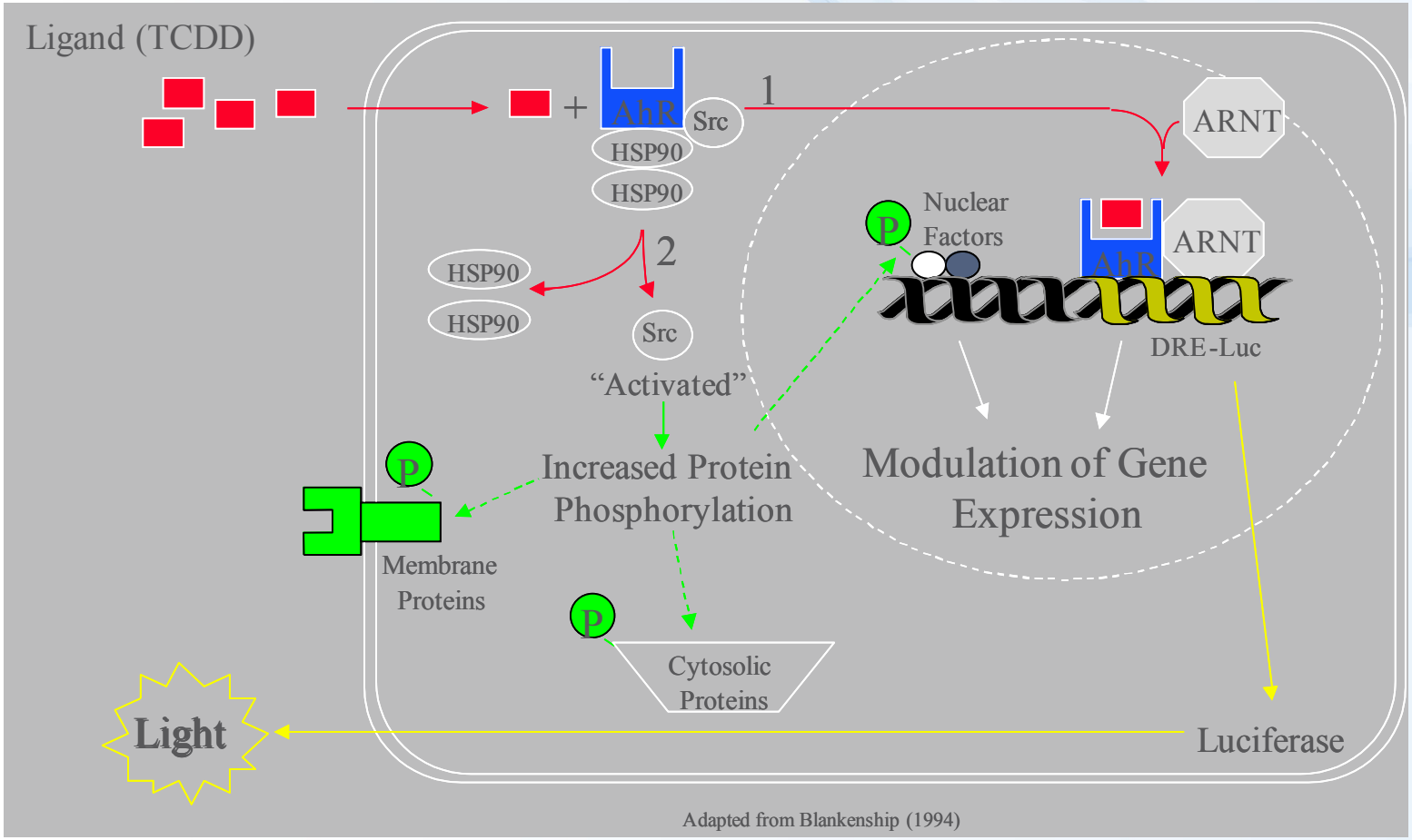
Biomarkers/bioanalytical methods for AhR toxicity

- In vivo studies
  - liver enlargement, reduction of thymus weight, wasting syndrome, reproductive and developmental disorders
- In vivo biomarkers
  - **EROD** activity, CYP 1A1 and 1B1 expression

- in vitro assessment of chemical potencies
  - EROD (ethoxyresorufin-O-deethylase activity) in cell cultures;
  - **CALUX/CAFLUX assays;**
  - GRAB assay (AhR-DNA binding)
  - yeast bioassay;
  - immunoassays;
  - detection of CYP1A mRNA (qPCR) or AhR protein (western blotting)

In vitro CALUX/CAFLUX assays

CALUX – Chemical Assisted Luciferase Expression
DR-CALUX (Dioxin Responsive CALUX)
(i.e. Luciferase Reporter Gene Assay with H4IIE.luc cells)



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DETECTION of EROD activity - example

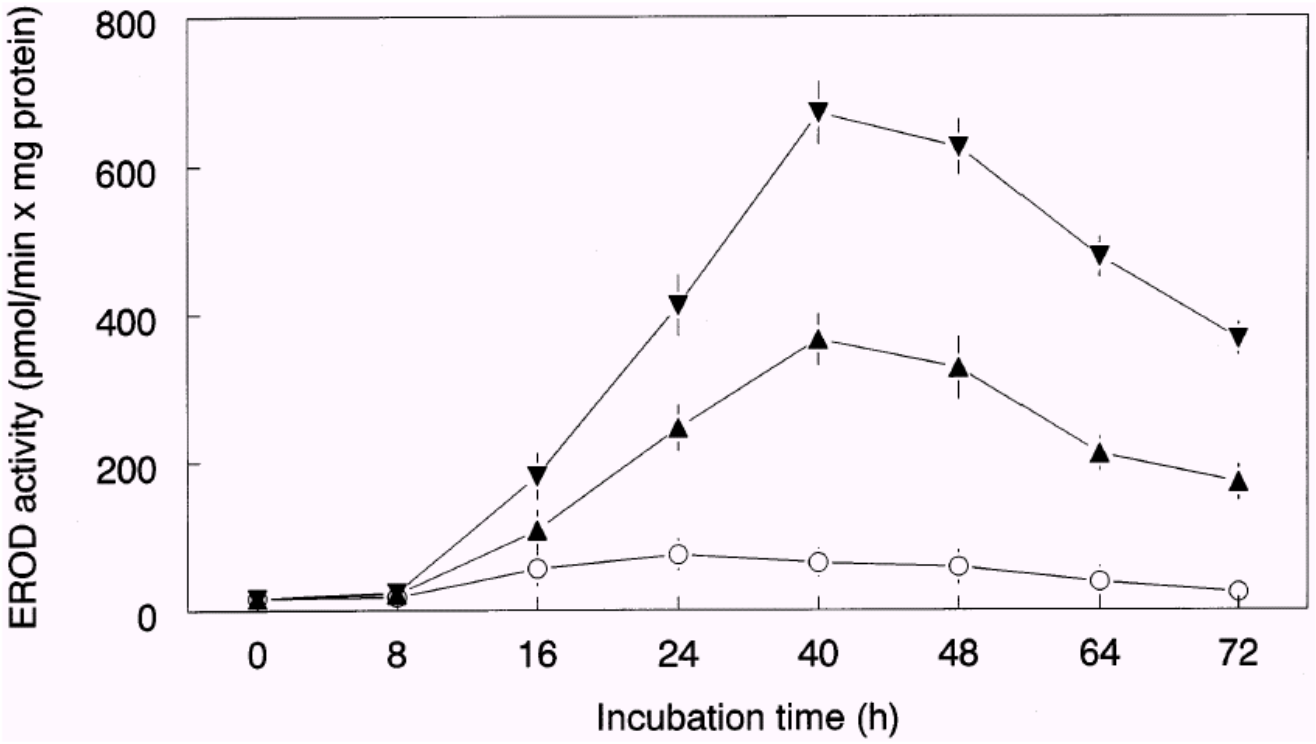
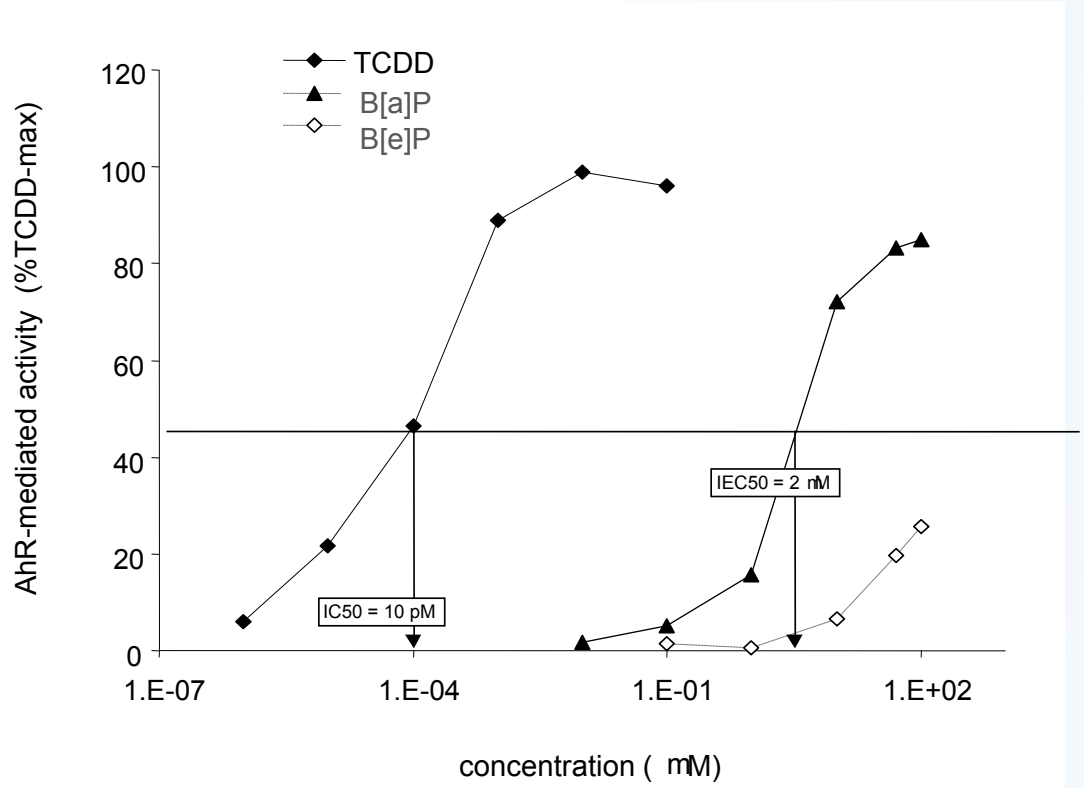


Fig. 2. Time course of induction of CYP1A1-catalyzed 7-ethoxyresorufin *O*-deethylase (EROD) activity in primary cultures of rat hepatocytes, after addition of 1.7×10^{-5} M benzo[*a*]pyrene (-▼-), 1.9×10^{-6} M benzo[*k*]fluoranthene (-▲-) or 9.4×10^{-5} M acenaphthylene (-○-). EROD activity was determined in cell homogenates. The data represent means ± S.D. from four independent experiments.

- Quantification of effects (EC_{50})
- Comparison with the effect of reference toxicant (2,3,7,8-TCDD)
  - → relative potencies (REPs) to TCDD
    (= in vitro "Toxic Equivalency Factors" ~ TEFs)



TCDD: IC_{50}
PAH: IEC_{50}

Relative Potency (REP)
= Induction Equivalency Factor

$$IEF = IC_{50} / IEC_{50}$$

REP interpretation: How many times is the compound "weaker" inducer than TCDD ?

Example - relative potencies of PAHs (two exposure periods)

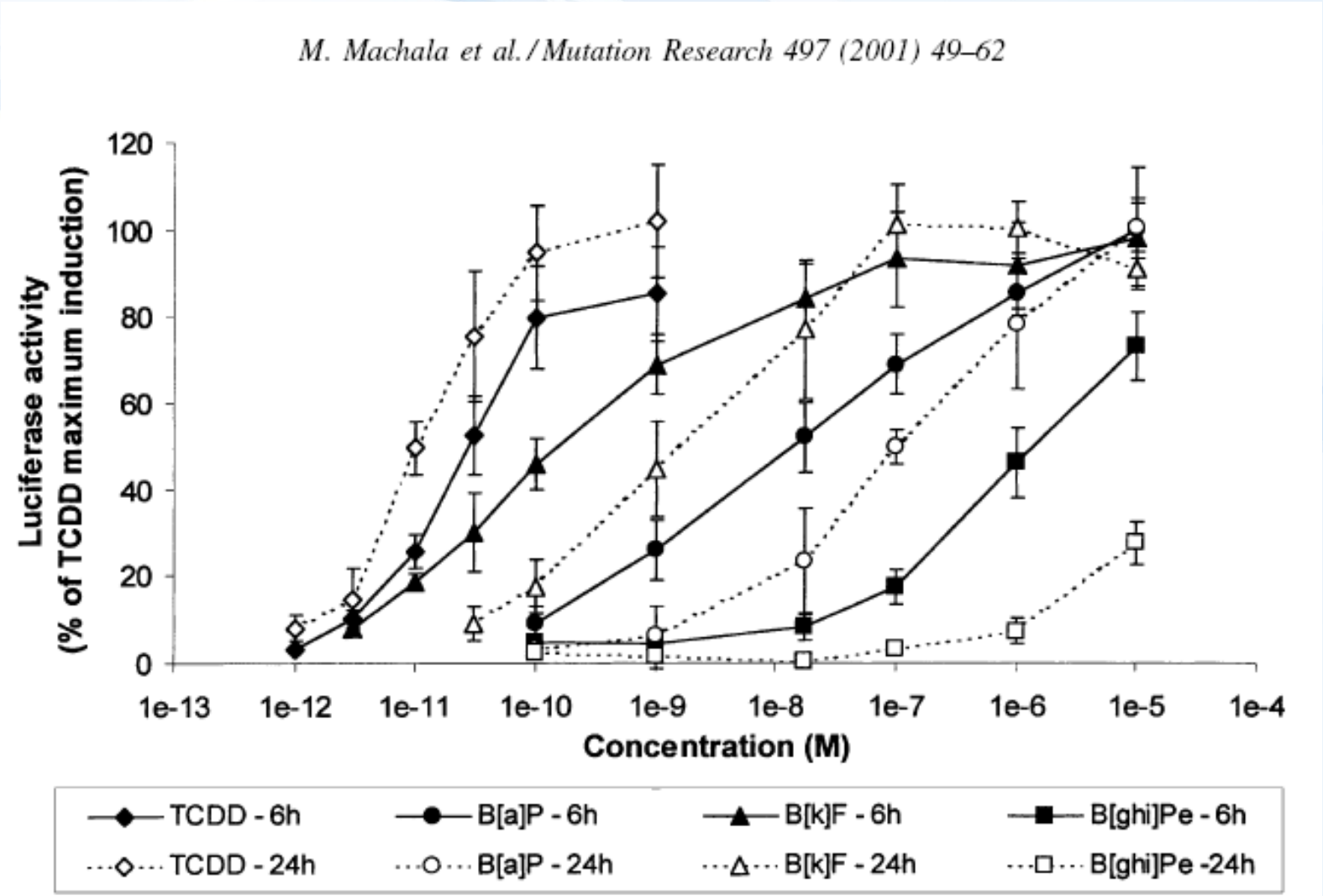


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Table 2
IEFs of PAHs relative to TCDD or B[a]P derived from EC50 or EC25 values in 24 and 6 h exposure assays

Derived from	$IEF_{TCDD(24h)}$		$IEF_{TCDD(6h)}$		$IEF_{B[a]P(6h)}$	
	EC50	EC25	EC50	EC25	EC50	EC25
Flu	ni[a]	ni	ni	ni	ni	ni
Ant	ni	ni	ni	ni	ni	ni
Fla	2.27E−8	9.31E−7	9.84E−5	1.11E−4	1.05E−2	5.59E−3
Py	1.78E−6	3.38E−6	2.59E−5	4.45E−5	7.57E−3	6.21E−3
B[a]A	7.04E−6	9.60E−6	7.64E−7	2.40E−6	0.39	0.50
Chry	1.01E−4	1.07E−4	1.41E−2	3.26E−2	3.25	2.04
B[b]F	3.35E−5	4.82E−5	4.90E−2	2.32E−1	8.83	12.81
B[k]F	1.64E−3	2.94E−3	0.28	0.57	67.76	36.33
B[a]P	9.01E−5	1.99E−4	1.11E−2	2.02E−2	1.0	1.0
DB[ah]A	1.17E−3	1.52E−3	0.06	0.20	11.46	11.72
I[123-cd]P	2.96E−4	5.01E−4	0.86	1.24	44.20	29.70
B[ghi]Pe	ni	ni	2.27E−5	4.68E−5	5.47E−3	2.99E−3
DB[al]P	4.90E−6	1.13E−6	2.52E−5	3.26E−5	2.36E−2	1.88E−2
NPyr	2.05E−4	3.83E−4	5.80E−3	1.31E−2	1.10	0.88
CPP	2.48E−7	6.53E−7	6.20E−6	1.72E−5	4.23E−3	3.38E−3
B[a]Pe	6.19E−6	6.28E−6	2.27E−4	3.05E−4	3.37E−2	1.68E−2
DB[ae]F	9.30E−6	1.18E−5	2.75E−5	1.33E−4	1.74E−3	6.74E−3
DB[ai]P	1.65E−4	4.41E−4	4.29E−2	3.82E−2	2.59	1.75
DB[ae]P	1.80E−5	3.90E−5	1.08E−3	3.90E−3	0.49	0.13
DB[ah]P	7.14E−5	3.70E−4	2.65E−2	5.43E−2	2.80	2.68
DB[ak]F	1.23E−3	1.37E−3	1.55E−2	2.02E−2	2.69	1.65
5-MeChry	9.48E−5	1.59E−4	4.05E−2	5.08E−2	3.07	2.46
DB[aj]A	3.70E−4	5.21E−4	3.07E−2	4.04E−2	2.16	2.16
B[j]F	3.68E−4	7.40E−4	4.05E−2	6.33E−2	2.25	2.51
B[c]Phe	4.49E−7	1.07E−6	6.21E−5	7.51E−5	4.64E−3	3.76E−3
B[e]P	5.15E−7	6.30E−7	3.71E−5	8.17E−5	2.27E−3	2.86E−3
DMBA	5.41E−6	1.30E−5	4.71E−2	3.98E−2	0.46	0.9
1-MePyr	2.07E−6	2.82E−6	4.80E−5	7.20E−5	8.54E−3	6.33E−3
DB[ac]A	1.92E−4	4.23E−4	3.53E−2	7.80E−2	1.75	2.78
Pic	4.11E−5	5.54E−5	1.90E−3	5.20E−3	0.12	0.25

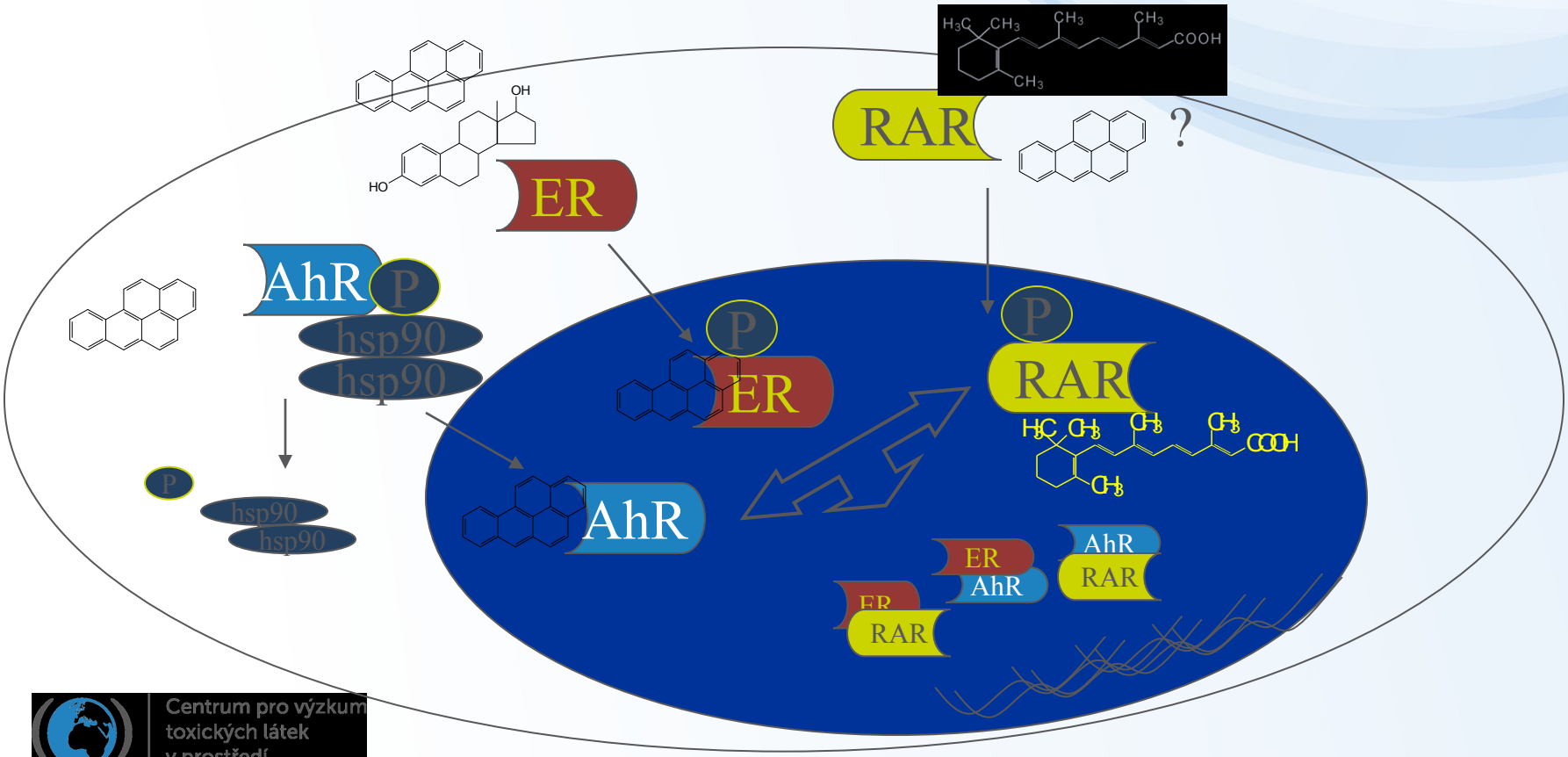
[a] ni, no induction observed.

Crosstalk in signalling of nuclear receptors

“Cross talk” of various NR → diverse toxic outcomes

Crosstalk effects documented but still less explored, e.g.

- “antiestrogenicity” of AhR ligands
- fast clearance of retinoids after AhR activation
- Immunosuprresions after ER activations



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Summary – Nuclear receptors

- Important physiological functions,
- Important roles in pathologies and chemical toxicity (ENDOCRINE DISRUPTION)

- NRs with well studied roles in toxicity: **ER and AhR**
  - Other NRs (AR, RAR/RXR, ThR) – important but less explored

- All NRs share similar structure and mechanisms of action
  - Act as direct transcription factors on DNA
- Natural ligands of NRs are small lipophilic hormones
  - steroids, thyroids, retinoids
  - Various regulatory functions
  - Role in toxicity: NR interact with **structurally similar xenobiotics**
- Various mechanisms beyond the toxicity
  - Adverse are both STIMULATIONS and INHIBITIONS directly at the receptor site (e.g. "anti-androgenicity)
  - Additional mechanisms – transport of hormones in blood (Thyroids), metabolism (Thyroids) clearance (Retinoids), heterodimerization and "crosstalk"

- Other key information to remember
  - REPORTER GENE ASSAYS (principle, use, what is CALUX?)
  - Characterization of chemical "toxic potentials"
    - General concept of "REPs" (valid for activation of all NRs)
    - Specifically for AhR - concept of TEFs / TEQs